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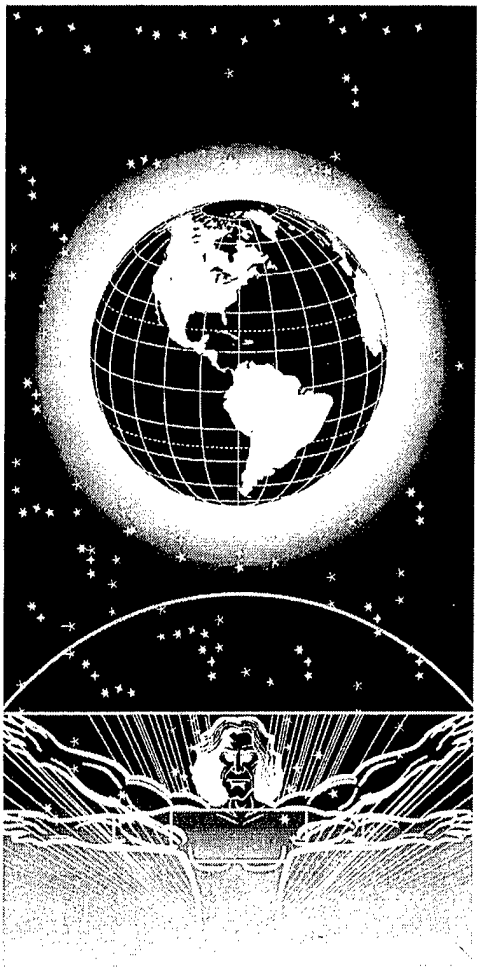

UNITED STATES AIR FORCE RESEARCH LABORATORY

CEREBRAL VASCULAR RESPONSES AND METABOLIC RAMIFICATIONS ASSOCIATED WITH +GZ-INDUCED LOSS OF CONSCIOUSNESS (G-LOC)

Asha R. Shahed, Ph.D.
Operatioanl Technologies Corporation
4100 NW Loop 410
San Antonio, TX 78229

HUMAN EFFECTIVENESS DIRECTORATE
BIODYNAMICS AND PROTECTION DIVISION
FLIGHT MOTION BRANCH
2503 Gillingham Dr.
Brooks AFB, Texas 78235-5102

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The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources – National Research Council.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the public, including foreign nationals.

This report has been reviewed and is approved for publication.

F. Wesley Baumgardner

F. WESLEY BAUMGARDNER, Ph.D.
Acting Chief, Biodynamics and Protection Division

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14. ABSTRACT
G-Induced loss of consciousness (G-LOC) can occur when an aircraft flies in a tight circular pattern for extended periods exposing pilots to high acceleration forces. Centripetal acceleration results in an inertial reaction of equal but opposite force (G) to displace organs and blood towards extremities. In humans (with or without G protection devices), G-LOC can occur at or above +5 Gz, and is characterized by ~15 s of absolute mental and physical incapacitation followed by equally long period of relative incapacitation. G-LOC occurs with a critical reduction in cerebral blood flow which has been measured in both baboons and human volunteers during 4+Gz and higher exposures in our centrifuge Even though the relationship between the reduction in CBF and +Gz exposure has been shown, little is known about its effect on brain physiology and metabolism. To address this problem a rodent-small animal centrifuge (SAC) model was developed at the Armstrong laboratory, Brooks Air Force Base, TX. The results of this unique and pioneering work are described in this report. This report describes the research conducted in the last three years on this contract. A major part of this research is presented as manuscripts of which some have been published, accepted for publication or have been submitted to journals for consideration. Also this research has been repented in many scientific meetings and abstracts of these presentations are also included. This report is organized in sections. Main objective of each section will be stated in the beginning and all results/publications/manuscript for that section will be presented.

15. SUBJECT TERMS
G- induced loss of consciousness, G-LOC, Brain Blood Flow, Cerebral Metabolism, Centrifuge, Acceleration

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Bibliography:

During the tenure of this contract the following publications in peer reviwed journals and abstract presentation at various national and international meetings were accomplished.

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4. Wiegman JF, Besch EL, Werchan PM, Nesthus TE, **Shahed AR**. Effect of Hypoxia and Hyperoxia on the physiologic response to anaerobic work. in preparation.

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SECTION 1

To study physiological and biochemical alterations induced by high $+G_z$ exposure in rat brain.

To accomplish this objective a rodent-SAC model was used and various +Gz exposure protocols were followed. A manuscript based on the findings of this research has been accepted for publication.

Manuscript # 1: Accepted for publication in the **Journal of Cerebral Blood Flow and Metabolism,** 1995.

Rat Brain Glucose and Energy Metabolites: Effect of $+G_z$ (head-to-foot inertial load) Exposure in a Small Animal Centrifuge (SAC)

A. R. Shahed, J. A. Barber[1], S. Galindo, Jr[2]., P. M. Werchan*
Operational Technologies Corporation, [1,2] Krug Life Sciences International, * Armstrong Laboratory, Brooks AFB.

Summary:

A unique small animal centrifuge (SAC) with online physiological monitoring and brain tissue collection (in < 1 s) capability was used to investigate the effect of increasing $+G_z$ levels, exposure duration, number of exposures and time course of metabolic changes in rat brain. To determine the $+G_z$ tolerance, rats were exposed to +7.5 to 25 G_z (2.5G increment) for 30 s each and EEG was monitored. Isoelectric EEG or loss of consciousness (G-LOC) occurred only at +22.5 and 25 G_z within 14.5 ± 3 s. To study the effect of increasing $+G_z$ levels on metabolism, several groups of rats were exposed to either 0.5 (control) or +7.5 to 25 G_z for 30 s. Brains were collected 1 min after the centrifuge run by freeze fixation. A significant increase in lactate ($\geq$ 7.5 G_z) and a decrease in glucose, Cr-P, and ATP levels was observed at $\geq$ 15 G_z. Rats were subjected to a single +22.5 G_z exposure for either 15, 30, 45 or 60 s to study the effect of $+G_z$ exposure duration. A six fold increase in lactate and a 75 % decrease in glucose was observed following the 60 s exposure. The concentrations of Cr-P and ATP decreased significantly after the 15 and 30 s exposures but no further changes were observed at longer $+G_z$ exposures. The time course of changes in these metabolites was determined by collecting brain samples at 5, 15, 25 or 35 s during or 1, 3, or 15 min after the $+25G_z$ exposure. A significant decrease in Cr-P occurred within 5 s, but changes in glucose, ATP and lactate required 15 s. The concentration of all metabolites returned to control levels within 3 min, except lactate and adenosine. Lactate returned to control levels and adenosine remained elevated 15 min post G exposure. Exposure of rats to either one, three or five runs at $+22.5G_z$ (30 s each) resulted in a nine fold increase in lactate and 87% decrease in glucose 1min after five exposures. Both Cr-P and ATP decreased after one exposure with no further change after 3 and 5 exposures. These results show that $+G_z$ exposure of short duration causes significant transient metabolic alterations consistent with global cerebral ischemia. We propose that G-LOC (isoelectric EEG) may be an adaptive response by the CNS to excessive $+G_z$-imposed ischemic stress. G-LOC would reduce the overall brain energy demand which in turn reduce anaerobic glycolysis and lactate production.

Introduction:

High sustained acceleration has been known to cause loss of consciousness (G-LOC) in pilots since World War (Burton, 1988). G-LOC can occur when an aircraft flies in a tight circular pattern for extended periods exposing pilots to high acceleration forces. Centripetal acceleration results in an inertial reaction of equal but opposite force (G) to displace organs and blood towards extremities. In humans (with or without G protection devices), G-LOC can occur at or above +5 G_z, and is characterized by ≈15 s of absolute mental and physical incapacitation followed by equally long period of relative incapacitation (Whinnery et al., 1987). G-LOC occurs with a critical reduction in cerebral blood flow which has been measured in both baboons and human volunteers during 4 $+G_z$ and higher exposures in our centrifuge (Werchan et al.,1989). Recently, using implanted flowprobes, we found a progressive decrease in carotid artery blood flow in rats during +5 to 25 G_z exposures (Werchan et al. 1993). A decrease in cerebral blood volume (CBV) in rats during $+G_z$ exposures, which caused G-LOC, has also been previously reported (Cananau and Ciuntu,1974; Galindo et al., 1992).

Even though the relationship between the reduction in CBF and $+G_z$ exposure has been shown, little is known about its effect on brain physiology and metabolism. Several studies (Lowry et al., 1964; Winn et al., 1979; Ekholm et al., 1992; Katsura et al., 1993) using decapitation, aortic transection or cardiac arrest rodent models have shown significant changes in glucose and energy metabolism within 30-120 s of global cerebral ischemia. Similarly, hypoxia of various degrees and duration has been shown to perturb brain energy metabolism (Duffy et al., 1972). Metabolically, the brain is a very active organ, uses ≈ 20 % of the total body oxygen consumption, and has relatively small substrate reserves. The CBF provides the main source of brain glucose and oxygen (Erecinska and Silver, 1989). Consequently, normal neuronal activity is completely dependent on a steady supply of ATP and decline in its concentration has been suggested for EEG changes (Lipton and Whittingham, 1982; Erecinska and Silver, 1989). Therefore, we hypothesized that $+G_z$ exposure-induced reductions in CBF would perturb brain glucose and energy metabolism and these alterations may be partially responsible for the occurrence of G-LOC.

In this study, we investigated the effect of increasing $+G_z$ levels, G exposure duration, number of repeated $+G_z$ exposures and time course of metabolic changes during and after $+G_z$ exposures in rats brains in a specially fabricated small animal centrifuge.

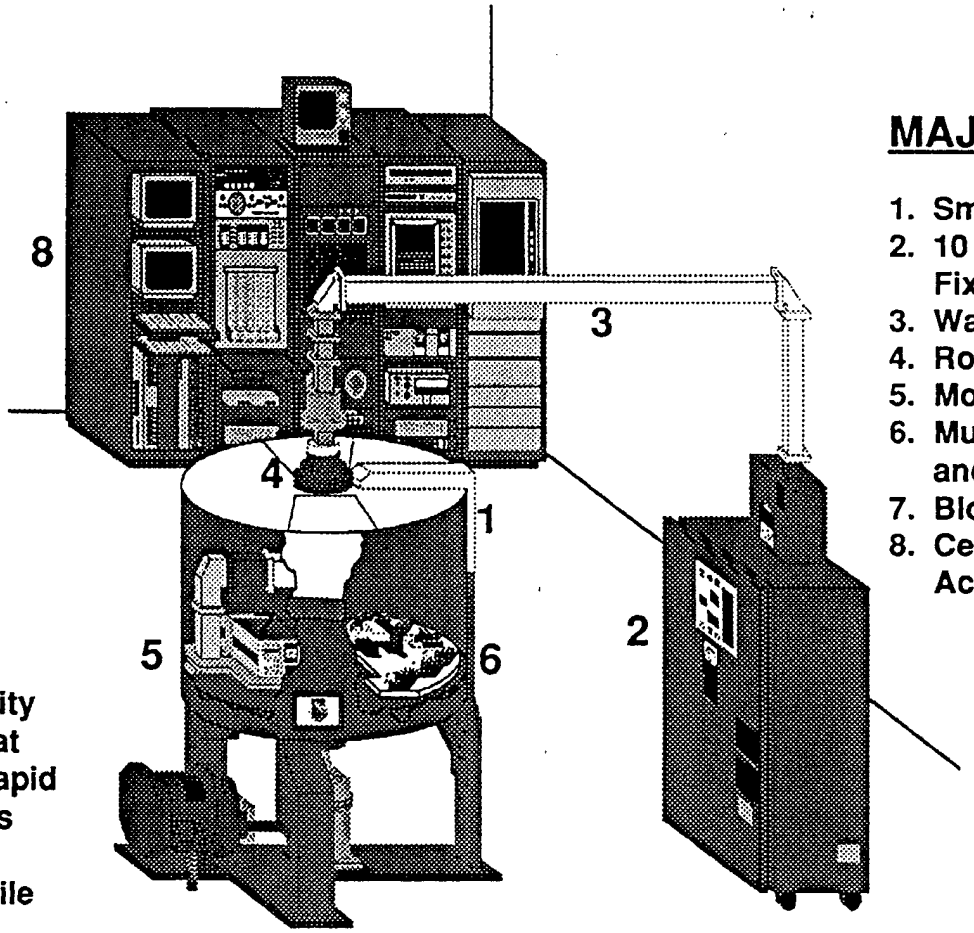
Experimental Methods:

Animal preparation: Adult male Sprague-Dawley CD-VAF / Plus rats (Charles River Laboratories, Wilmington, MA) were provided free access to food and water. Biparietal lateral EEG electrodes were implanted using two #0-80 stainless steel screws with the rat under halothane anesthesia. A third screw (ground) was placed slightly cranial to the bregma suture and lateral to the midsagittal suture (Werchan and Shahed, 1992). All screws and attached wires were imbedded in cranial plastic cement and the wound site closed. Two to 3 h after surgery, when the rats were fully awake, they were placed in a Plexiglas holder and clamped to the SAC arm (without causing pain and discomfort) so the head faced the center shaft of the centrifuge for a $+G_z$ exposure.

Small Animal Centrifuge (SAC): The SAC (Fig. 1) is 5 ft (1.52 m) in diameter and is powered by a 15 hp three phase regenerative direct current (DC) drive motor and is capable of 1-85 $+G_z$ with an onset/offset rate of 20 $+G_z$/s (Werchan and Shahed, 1992, Shahed et al., 1994)). The operation of the SAC is controlled by specialized software linked to an optical encoding sensor. The SAC is equipped with a video camera, syringe pump, pressure transducers and amplifiers for monitoring EEG and ECG. Eight channels of data can be collected and stored with a

AL SMALL ANIMAL CENTRIFUGE

Figure 1



SPECIFICATIONS

- 5 ' diameter, 5 ' height
- Gz, Gy and Gx acceleration vectors
- 85 G max limit, 13 G/sec onset rate
- Slip ring assembly linked to physiological data acquisition system
- Complex G profile capability
- Freeze and microwave heat inactivation systems for rapid "fixation" of brain samples at any timepoint before, during or following G profile

MAJOR FEATURES

1. Small Animal Centrifuge
2. 10 KW (2450 KHz) Microwave Fixation System
3. Wave Guide
4. Rotary Waveguide Joint
5. Mouse Microwave Applicator
6. Multi-rat (7) restraint system and freeze fixation device
7. Blood Flowmeter
8. Centrifuge Control and Date Acquisition Systems

Macintosh IIfx computer for either online or subsequent EEG analysis. The SAC is equipped with a freeze fixation device that injects compressed air (35 psi) into the rat cranium to homogenize, remove and freeze brain tissue (Veech, et al. 1973). Approximately 75 % of the brain can be collected between two aluminum disks precooled in liquid N_2 in less than 1 s. Most importantly, the freeze fixation device can be triggered when the SAC is either in motion or static.

+G_z- Exposure Protocols: In all protocols an onset/offset rate of +20 G_z /s was used and brain samples were collected at indicated time points by freeze fixation. The control group(n=6) was exposed to +0.5 G_z for 30 s, and brain samples were collected 1 min after the run (protocols 1, 2 and 4) or at 30 s during the run (protocol 3) by freeze fixation.

Protocol 1: Effect of +G_z exposure on EEG and brain metabolites: Six groups (n=6 in each group) were exposed to a single 30 s exposure of either +7.5, 10, 15, 17.5, 20, 22.5 or 25 G_z, and brain samples collected 1 min after the run.

Protocol 2: Effect of +G_z exposure duration on brain metabolites: Four groups of rats (n=6 in each group) were exposed to +22.5 G_z for either 15, 30, 45 or 60 s and brain samples were collected 1 min after the run.

Protocol 3: Time course of metabolic changes before, during and after a single +25 G_z exposure: Four groups of rats (n=6 in each group) were exposed to +25 G_z for either 5, 15, 25 or 35 s, and brain samples were collected at these time points with the centrifuge running. In addition, three groups of rats (n=6 in each group) were exposed to +25 G_z for 35 s and brain samples were collected either at 1, 3 or 15 min following the run.

Protocol 4: Effect of multiple +G_z exposures on brain metabolites: Three groups of rats (n=6 in each group) were subjected to 1, 3 or 5 exposures at +22.5 G_z for 30 s each with a 30 s interval between each run, and brain samples were collected 1 min following the last exposure.

Assay of Metabolites: The frozen brain tissue (0.2g) was homogenized in 2 ml of 7% cold perchloric acid using a Polytron homogenizer and centrifuged for 10 min at 10, 000 rpm. The supernatant was neutralized with KOH. Lactate, pyruvate, and glucose were assayed as previously described (Shahed et al., 1994). An aliquot (100 to 200 µl) of the supernatant was filtered (0.45 µm) and creatine phosphate (Cr-P) and adenine nucleotides were measured by ion pair reversed phase high performance liquid chromatography (HPLC) on a Waters NOVA PAK C_{18} column. A linear gradient of mobile phase A (50 mM potassium phosphate and 3 mM tetra butyl ammonium phosphate pH 5.8), and mobile phase B (mobile phase A +15 % acetonitrile) was used. Inosine and adenosine were measured using a linear gradient of 100 mM potassium phosphate + 1% methanol (pH 4.5) and 100 mM potassium phosphate + 25 % methanol (pH 5.3). Protein was measured using a BCA Protein Assay Kit (Pierce, Rockford Ill).

Statistics: The data were analyzed by one-way analysis of variance (ANOVA) and Dunnett's follow-up test for multiple comparisons. All data are presented as means ± SD of six animals.

Results:

Effect of increasing level of +G_z exposure on EEG and brain metabolites (Protocol 1): During 0.5, 7.5, 10 and 12.5 +G_z exposures no alterations in EEG recordings were observed (data not shown). However, EEG patterns started to alter during the +15 to 20 G_z exposures, and became flat or near isoelectric at 22.5 and +25 G_z. The isoelectric EEG or G-LOC was observed 14.5 ± 3 s after the onset of +22.5 G_z. EEG remained isoelectric for an additional 15 to 45 s after the termination of the centrifuge run (Fig. 2).

The effect of increasing $+G_z$ levels on brain metabolites is shown in Figs. 3a and 3b. Control rats, which underwent identical surgical procedures as the experimental group (EEG electrode implantation), were exposed to +0.5 G_z in the SAC for 30 s to incorporate stresses imposed by restraint and noise. A significant increase in lactate production was observed at and above +7.5 G_z. A decrease glucose level became significant only at and above +17.5 G_z (Fig. 3a). Significant decreases in the concentration of creatine phosphate (Cr-P) and ATP were observed at or above the +15 G_z exposure (Fig. 3b). The levels of AMP, but not ADP, were elevated at and above + 7.5 G_z (Fig. 3b). Generally, the changes in these metabolites plateaued at or above +15 G_z exposure.

Effect of $+G_z$ exposure duration on brain metabolites (Protocol 2): For these experiments the +22.5 G_z level was selected since this was the minimum level to induce G-LOC. Control rats were exposed to + 0.5 G_z for only 30 s since preliminary experiments revealed no significant difference in the level of metabolites between 30 and 60 s runs. There was no significant change in tissue glycogen but the concentration of glucose decreased significantly by 38, 79 and 75% respectively after 30, 45 and 60 s exposures (Table 1). Two, four, five and six fold increases in lactate levels were observed as exposure durations increased from 15 to 60 s. The concentration of Cr-P and ATP decreased by 63% and 33% respectively after 15 s, remianing relatively constant with longer $+G_z$ exposure duration. The concentration of AMP increased significantly, whereas the ADP, adenosine and inosine levels were not different from control as the exposure duration increased.

Time course of metabolic changes before, during and after a single +25 G_z exposure (Protocol 3): In the above experiments, metabolites were measured in brain samples collected 1 min after the termination of the SAC run. In the time course experiment brain samples were collected at 5 s, 15 s, 25 s, and 35 s, while the SAC was still running and 1, 3 and 15 min after the run was abolished. The concentration of lactate increased and glucose decreased significantly 15 s after the onset of $+G_z$, and continued to change at 25 s, but tended to plateau by the 35 s time point (Fig. 4a). Glucose and lactate levels returned to control levels, 3 and 15 min after the SAC run, respectively. A decrease in the concentrations of Cr-P (21, 52, 62 and 65%) and ATP (12.5, 50, 47, and 60%) was observed at 5, 15, 25 and 35 s time points respectively (Fig. 4b). The decrease in ATP was not significant until the 15s time point. The rate of decrease in Cr-P and ATP, like lactate, appears to plateau after the 15 s time point, which is coincidental with the occurrence of G-LOC. The concentration of Cr-P and ATP returned to control level 3 min after the SAC run (Fig. 4b). The concentration of adenosine increased steadily during $+G_z$ exposure, but a much greater elevation was observed at 1 and 3 min, and it remained significantly elevated 15 min following the termination of the SAC run (Fig. 4b).

Effect of multiple $+G_z$ exposures on brain metabolites (Protocol 4): The time for the appearance of isoelectric EEG or G-LOC was not significantly different during 1, 3 and 5 $+G_z$ exposures (15.5 ± 4, 13.4 ± 3.2 and 20.3 ± 7 s respectively). However, the EEG recovery times (lapse for EEG to become normal) were delayed with increasing number of exposures eg. 27.5 ± 6, 39.4 ± 11, and 42.5 ± 14 s for the 1, 3 and 5 exposures respectively. After five consecutive 22.5 $+G_z$ exposures a significant depletion of glucose (87 %) and a 9 fold increase in lactate accumulation was noted (Table 2). Although a significant decrease in Cr-P and ATP was observed after a single exposure, no further change was noted after 3 and 5 exposures. The concentration of AMP increased about four fold and three fold after one and three exposures respectively, but it was not significantly different from the control after 5 exposures. Changes in ADP concentrations were not systematic.

Discussion:

G-LOC, a potentially deadly situation, arises when an aircraft flies in a tight circular pattern for an extended period of time. It has long been theorized that the centripetal acceleration produced in the tight turns results in an inertial reaction of equal but opposite force (G) to displace organs and blood towards the feet. Over the last 60 years many human centrifuges have been built and dedicated to the research of the complex physiology imposed by the high $+G_z$ environment. This research led to the development of the anti-G straining maneuver and the anti-G suit to increase $+G_z$ tolerance of pilots of high performance aircraft. However, the problem of G-LOC still exists despite use of anti -G suit and anti-G straining maneuver and potentially could become greater in the future as advances are made in the aircraft industry to enhance maneuverability. Although systemic cardiovascular physiology has been thoroughly investigated, little or no research has focused on the brain, the main target organ during the acceleration insult. This is primarily due to the lack of tools and techniques necessary to accomplish this goal. It has been hypothesized that G-LOC is the manifestation of either a total or incomplete cessation of blood flow to the brain. Thus the metabolic consequences should be similar, if not identical, to the changes identified using other models of cerebral ischemia. An understanding of the complete mechanism of G-LOC is essential for the development of therapeutics to increase G-tolerance and prevent G-LOC. To aid discussion, a schematic drawing/model of the proposed G-LOC mechanism based primarily on the findings presented in this paper and previous studies is provided (Fig.5).

The loss of CBF in both high G pilots and the rodent model may be due to the combination of two distinct cardiovascular events. If the onset of G is rapid and the level of G is sufficient then the inertial force will exaggerate the normal hydrostatic column gradient from the heart to the brain. This relationship can be expressed in the following formula (Burton, 1973; Burton et al., 1991):

$$G = Pa \times (d/h)$$

where Pa is the mean arterial blood pressure,
h is the height (in mm) of the fluid column (heart-to-eye distance),
d is the density of blood related to the specific density of mercury, and
G is the inertial force of acceleration exposure

This formula was used to estimate the G-tolerance of rats to be 27.2 $+G_z$, based on a heart-to-eye distance of =50mm, Pa of 100 mm Hg and d=13.6. In the present study, the $+G_z$ tolerance of rats was found to be 22.5 $+G_z$, which correlates well to the calculated value and is about 50% of the LD_{50} level which has been reported previously (Chae. 1975).

In contrast, when the onset rate of G is low, or the duration of G exposure is extended, then venous return to the heart could be severely compromised as blood begins to pool in the lower extremities. This situation could be particularly important in the rodent model since rats are not protected (as by G suits in pilots) to minimize blood pooling. The decrease in venous return could cause reduction in cardiac output. Both pathways of systemic cardiovascular effects will result in decreased eye level blood pressure instantaneously after the onset of $+G_z$. This situation has been observed in humans (Henry et al., 1951; Lambert and Wood, 1943) and in swine (Burton, 1973). On the other hand, if the G onset rate is high, a drop in Pa may not immediately influence CBF if autoregulatory mechanisms are invoked (see Fig. 5). However, studies from this laboratory have shown a 95% reduction in the carotid artery blood flow (using a chronically implanted carotid artery flowprobe) and a 45% reduction in CBV in rats 5 s after the onset of a +25 G_z exposure (with 20 G/sec onset) in the SAC (see Introduction). Studies on CBF using different G onset rates and exposure duration are in progress.

Although, the reduction in CBF occurs within seconds after the onset of +22.5 G_z, G-LOC in these rats did not occur until about 15s, similar to that previously reported for humans (Jasper et al., 1945). The reason for this delay is not fully clear, but based on the results of this study it is suggested that the level of oxygen and energy reserves of the brain may be sufficient for maintaining neuronal activity during the 15 s period prior to the onset of G-LOC (Figs. 3a, b, 4a and b). We have previously reported that eventhough CBF stops with in seconds of onset of $+G_z$, CBV decreases only by 45% which may be the result of "trapped" blood in the capillaries (Galindo et al., 1992). In addition, it is possible that hemodynamic compensatory factors, such as cerebral vasodilation (Henry et al., 1951) and a negative cerebrospinal fluid pressure (Moskalenko et al., 1974; Rushmer, Bechman, Lee, 1947) may play a role in maintaining the intracerebral circulation at a very low level during $+G_z$ exposure. The data in Fig. 4b show an increase in adenosine concentration during and after $+G_z$ exposure. Since adenosine (a degradation product of ATP) is a potent cerebral vasodilator (Wahl and Kuschinsky, 1979; Winn et al. 1979) it is suggested, that it may play a role in the delay of G-LOC and may also be especially important during the recovery phase after the $+G_z$ exposure.

Relatively little is known of the effect of short duration high $+G_z$ exposures on brain metabolism. Accomplishment of this goal required the development of a device to rapidly stop all brain enzymatic reactions at any timepoint before, during, or following a high $+G_z$ exposure in the centrifuge. Two tissue fixation devices namely freeze fixation and microwave fixation were incorporated in the SAC allowing brain tissue collection in less than 1s. The microwave fixation unit (2450 Mhz, 10 Kw, New Japan Radio Inc.) is used for the study of regional brain metabolism in mice. Both of these units are fully operative during the centrifuge run and can be triggered by customized computer software at any time.

In previous studies (Debiec et al., 1978; Cananau et al., 1975) rats were exposed to 10 $+G_z$ (0.1 $+G_z$/s) for 3 to 60 min and only glucose and lactate levels were measured in brain samples collected long after the centrifuge run. Under these conditions, a significant increase in brain glucose and lactate concentration and a decrease in the activity of certain cytosolic and mitochondrial enzymes were reported. In the present study, rats were exposed to increasing $+G_z$ levels using a onset rate of +20 G_z/s, for short (30 s) duration; glucose and energy metabolites were measured both during and 1 min following the SAC run. The results clearly show a significant increase in lactate production starting at only +7.5 G_z, whereas reductions in glucose, Cr-P and ATP were seen at or above +15 G_z (Figs. 3a and 3b). These alterations are probably the result of partial to complete cerebral ischemia and a subsequent shift to anaerobic glycolysis. It is likely that a complete cessation of CBF occurs at or above +22.5 G_z, resulting in G-LOC. It is also notable that the rate of metabolic changes appears to plateau beyond the +15 G_z exposure (Fig. 3a and 3b). This may be the result of reduced neuronal activity as reflected in EEG changes (Fig.2) during >+15Gz exposures, which in turn reduces energy demand and anaerobic glycolysis. This hypothesis is further supported by data in Table 1. The increase in the +22.5 G_z exposure duration from 15 to 60 s, contrary to our expectation, did not result in greater proportional changes of Cr-P, ATP or lactate. For example, there was a 91 and 111% increase in lactate production after the 15 and 30 s exposures respectively but only a 22 and 23% increase (over 30 s time point) was noted after 45 and 60 s exposures. These kinetics suggests a slowing of the metabolic rate.

The timecourse of metabolic changes during a single +25 G_z exposure (Figs. 4a and b), for the most part, are similar to those observed using bench top models of sudden complete global ischemia. Based on this similarity, and our earlier studies showing reductions in both CBF and

CBV, it is suggested that a +25 G_z exposure may induce complete global ischemia for approximately 30 s. In the rodent-SAC model significant decreases in Cr-P and ATP (50%) levels were seen within 5 and 15 s respectively (Fig. 4b). However, in static models, a significant decrease in ATP levels has been shown only after 60 s of complete global ischemia in anesthetized rats (Winn et al., 1979; Ekholm et al., 1992). This difference could be due to the fact that our centrifuge rats were fully awake. Depletion of energy metabolites has been proposed as one of the reasons for the cessation of neuronal activity (Siesjo, 1978; Raichle, 1983); however, several studies have shown that EEG becomes isoelectric within 15 s after ischemia without a significant decrease in ATP level (Naritomi et al., 1988; Alger et al., 1989). In the present study, although significant decreases in the concentrations of Cr-P (57%) and ATP (50%) were observed, they were not depleted at the onset of isoelectric EEG (Fig. 4b). Also, after the SAC run and presumably the resumption of CBF, EEG recovery was evident within 15 to 45 s, but Cr-P and ATP levels were not restored to control levels until 3 min. This disparity may be partially explained using the concept of energetic compartmentation which suggests that the location and rate of ATP utilization may be heterogeneous within the nerve cell (Whitingham, 1990; Erecinska and Silver, 1989). That is, during ischemia ATP may be depleted more rapidly in one area of the cell than in others, and this (depletion) may not be fully reflected when whole brain concentrations are measured as was done in the present study. Neuronal function depends upon the preservation of an ionic gradient across the cell membrane and it is estimated that about 50% of ATP is used for this purpose by the activity of Na+-K+ATPase (Astrup, 1981). Since a 50% decrease in ATP (Fig.4b) was observed at the time of G-LOC (15 s post onset of $+G_z$), it is hypothesized that high $+G_z$ exposure causes global cerebral ischemia and a subsequent decrease in energy production/reserve (below the critical level in discrete areas), which may trigger the loss of neuronal activity of G-LOC.

Several studies using either humans (Burton, 1980) or animals (Borredon et.al., 1985) have investigated the effect of repeated $+G_z$ exposures. An increase in blood lactate concentration and creatine kinase MM activity was reported following repeated $+G_z$ exposures in human subjects (Burton, 1980). Recently, multiple $+G_z$ exposures have been shown to cause brain edema in rats (Shahed et al., 1994). The effect of multiple +Gz exposures on cerebral metabolism is not known. Contrary to our expectation the levels of Cr-P and ATP were not depleted as the +22.5 G_z exposures (ischemic episodes) increased from 1 to 5 (Table 2). It is likely, that during the 30 s rest period between runs (where $+G_z$ level returned to +0.5) a post-exposure hyperemic response (Werchan et al., 1989, 1993) partially replenished glucose and oxygen and prevented a further reduction of Cr-P and ATP. Also, since the time for an animal with an isoelectric EEG (G-LOC) to return to normal increases with the number of G-exposures, it is likely that during the 3rd and 5th $+G_z$ exposures neuronal activity is still depressed, and thus the energy demand remains low. Although, the concentration of glucose, after three exposures, was similar to the controls, it was 87% lower and the level of lactate was more than 9 fold higher after five exposures (Table 2). It is speculated that following five successive exposures, the cardiovascular system is fatigued and a reduced cardiac output, could minimize the post $+G_z$ exposure hyperemic response. This could result in a mismatch of supply and demand of tissue glucose. The increased accumulation of lactate is not surprising since these measurements were made 1 min following the SAC run, and the time course studies show that 15 min are required for lactate levels to return to control levels (Fig. 4a). These results suggest that repeated G-LOC episodes may be more severe due to cardiovascular changes and lactoacidosis.

In summary, our study shows that the rodent-SAC model is ideal for investigating the physiologic and metabolic effects of $+G_z$ exposure leading to G-LOC. Furthermore, high $+G_z$ exposures, as short as 15 s, cause significant but transient alterations in CBF and cerebral

metabolism consistent with global ischemia and resulting in G-LOC. It is proposed that G-LOC could serve to reduce energy consumption (due to loss of neuronal function) and therefore reduce the need for anaerobic oxidation of glucose which produces lactate

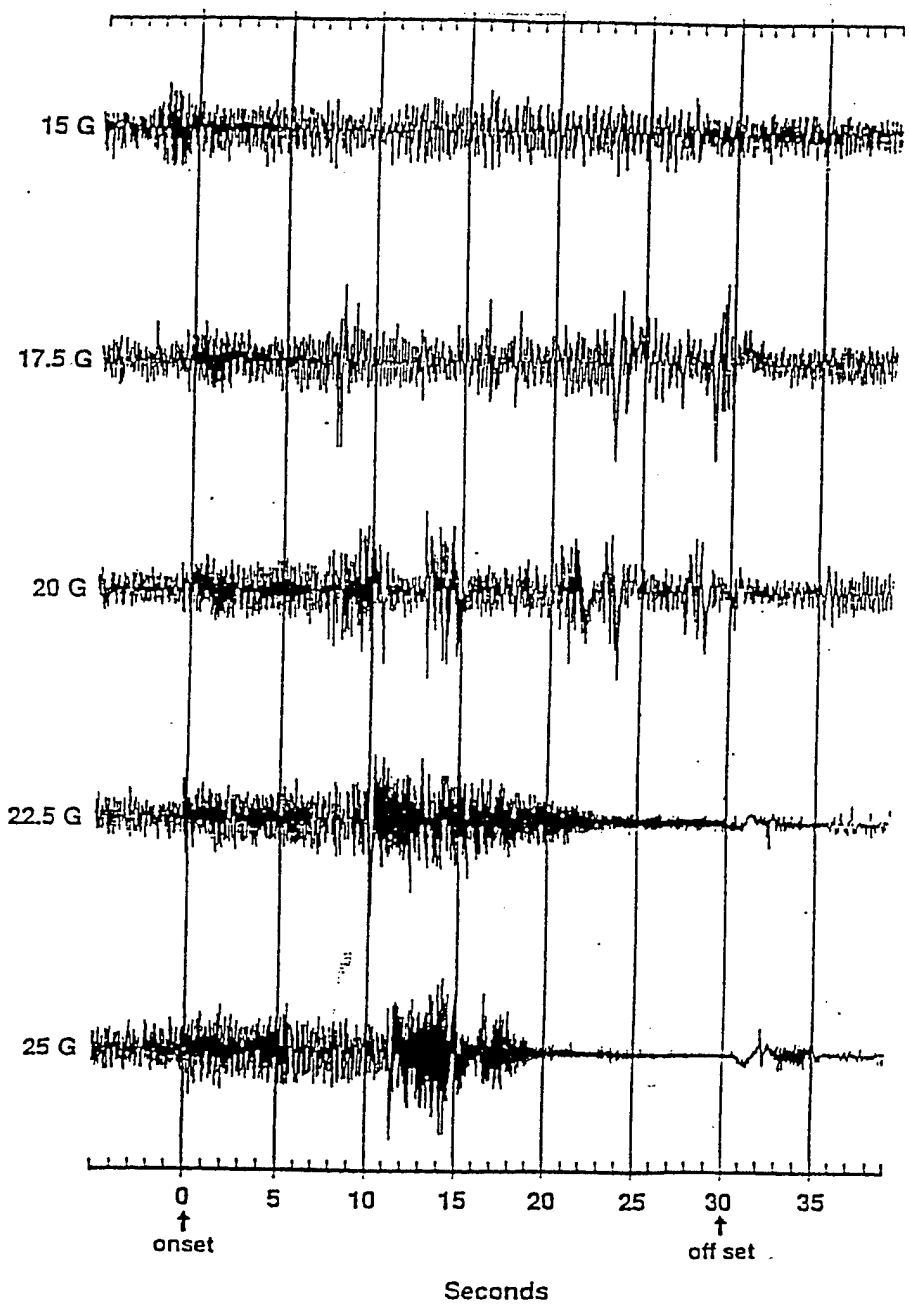


Figure 2: Effect of increasing $+G_z$ levels on rat EEG. Biparietal lateral EEG electrodes were implanted under halothane anesthesia as described under methods. Fully awake rats were subjected to indicated $+G_z$ levels for 30 s (Protocol 1) and EEG was monitored.

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Table 1
Effect of $+G_z$ Exposure Duration on Brain Glucose and Energy Metabolite

Metabolite	Control	15 s	30 s	45 s	60 s
Glycogen	1.96 ± 0.9	1.38 ± 0.7	2.81 ± 1.3	1.90 ± 0.3	2.02 ± 0.2
Glucose	2.65 ± 0.9	2.73 ± 0.9	1.64 ± 0.5*	0.55 ± 0.2*	0.65 ± 0.3*
Lactate	1.31 ± 0.4	2.51 ± 0.2*	5.31 ± 1.2*	6.48 ± 0.8*	7.99 ± 0.8*
Cr-P	3.90 ± 0.7	1.44 ± 1*	2.01 ± 0.5*	1.91 ± 1.4*	1.88 ± 0.1*
AMP	0.26 ± 0.1	0.70 ± 1 *	0.97 ± 0.3*	0.63 ± 0.2*	0.57 ± 0.1*
ADP	0.41 ± 0.1	0.46 ± 0.07	0.44 ± 0.16	0.48 ± 0.14	0.54 ± 0.04
ATP	2.10 ± 0.26	1.33 ± 0.2*	1.45 ± 0.4*	1.02 ± 0.8*	1.55 ± 0.4*
Adenosine	9.08 ± 2.6	12.03 ± 4	18.26 ± 7*	6.49 ± 3.8	6.42 ± 4
Inosine	18.60 ± 5.5	12.81 ± 7	20.86 ± 12	49.83 ± 43	24.83 ± 6.4

Mean ± SD, *P < 0.05, as compared with control, n=6, at each time point. Values are expressed as µmol/g wet wt of the tissue, except for adenosine and inosine (nmol/g wet wt). Rats were exposed to +22.5 G_z for 15, 30, 45 or 60 s in the SAC (Protocol 2) and brain samples were collected 1 min after deceleration by freeze fixation. Control rats were exposed to +0.5 G_z for 30s.

Table 2
Effect of Multiple $+22.5G_z$ Exposures on Brain Glucose and Energy Metabolites

Metabolites	Control	1 Exposure	3 Exposures	5 Exposures
Glycogen	1.96 ± 0.9	2.81 ± 1.3	1.20 ± 0.6	1.75 ± 0.3
Glucose	2.65 ± 0.9	1.64 ± 0.5*	2.61 ± 1	0.34 ± 0.3*
Lactate	1.31 ± 0.4	5.31 ± 1.2*	8.74 ± 0.7* ¥	11.08 ± 1.2*¥
Cr-P	3.90 ± 0.6	2.01 ± 0.5*	2.55 ± 0.7*	2.32 ± 0.8*
AMP	0.26 ± 0.1	0.97 ± 0.28*	0.82 ± 0.24*	0.53 ± 0.26
ADP	0.41 ± 0.14	0.44 ± 0.16	0.64 ± 0.17*	0.52 ± 0.26
ATP	2.10 ± 0.26	1.45 ± 0.41*	1.38 ± 0.5*	1..38 ± 0.25*

Mean ± SD. * P< 0.05 as compared to control, ¥ P< 0.05 as compared to 1 exposure values, n=6 in each group. Rats were subjected to one, three or five 30 sec exposures at +22.5 G_z, with 30 sec rest period between each run, and brain samples were collected 1 min after the last run by freeze fixation (Protocol 4). Control rats were subjected to one 30 sec run at + 0.5 G_z.

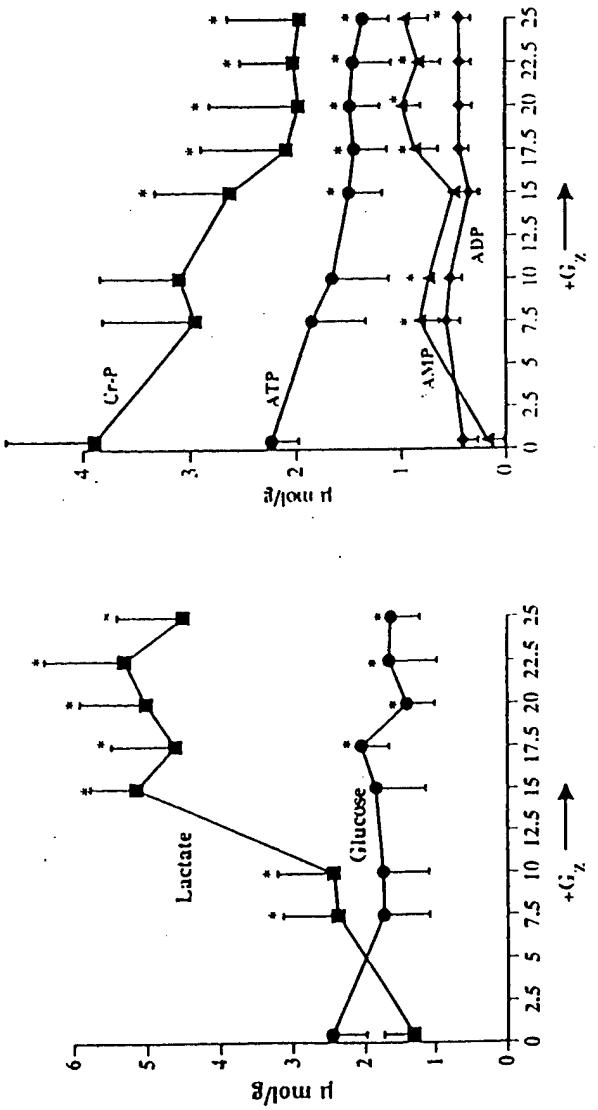
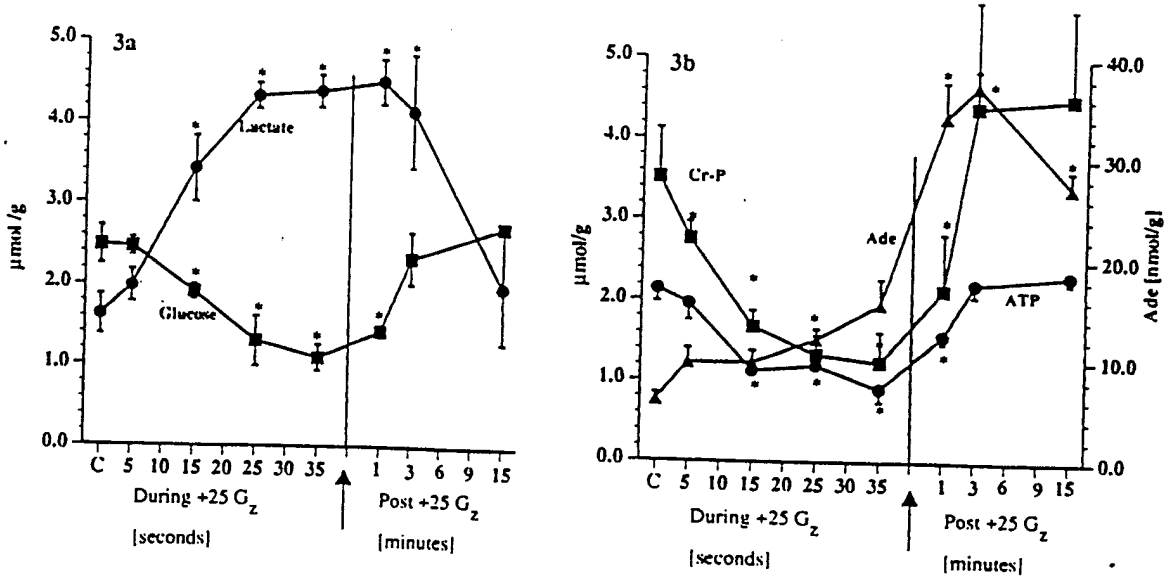


FIG. 3 Effect increasing $+G_z$ stress on glucose and lactate. Mean ± SD, *$p < 0.05$, as compared with control. Rats were exposed to each $+G_z$ level for 30 s (protocol 1), and the brains were collected 1 min postcentrifugation by freeze fixation.

FIG. 4 Time course of metabolites (protocol 3). Mean ± SD (n = 6/time point), *p < 0.05 as compared with control. Solid vertical line indicates change in the scale. Rats were exposed to +25 G_z for 5, 15, 25, or 35 s, and brains were collected while the centrifuge was still running. Three more groups of rats were exposed to +25 G_z for 35 s, and brains were collected 1, 3, or 15 min postcentrifugation.



SECTION 2

To investigate the effect of $+G_z$ onset rate on G-LOC and brain metabolism.

Experimental Protocol:

All rats were instrumented with EEG electrodes (see above for surgical procedures). Rats were exposed to two exposures of +25 G_z until G-LOC was observed using 0.5, 1.0, 2.0 and 10.4 +Gz onset rates in the SAC. During the first exposure EEG was recorded before, during and after G-LOC. The second exposure, after a 15 min rest period was exactly like the first but the brain sample was collected at the time of G-LOC by freeze fixation. EEG data was analyzed for G-LOC induction and recovery times. Brain samples were analyzed for glucose and energy metabolites as described above.

Results:

The data (Fig. 1) showed that onset rate had no significant effect on G-LOC induction time. G-LOC in all group was observed in less than 12 s after reaching +25 G_z. The time to reach +25Gz was different at different onset rates eg. 50 s, 25, 12.5 and 2 s at the onset rates of 0.5, 1, 2 and 13 G/s. There was no significant differences in EEG recovery times between different onset rates. In addition there was no significant differences in the decrease in cerebral blood volume with the onset rates. However, significant differences were observed in lactate production. The highest increase was seen at 0.5G/s >1.0 >2.0 and 10.4G/s. All lactate levels were higher than control. In contrast the decrease in ATP and creatine phosphate was lowest at the 0.5G/s onset rate.

Conclusions:

It was concluded that physical stress generated by different G onset rates does not contribute to the onset of G-LOC. Also that increased lactate production at the low onset rates does not contribute to the onset of G-LOC. However it is possible that low onset rates may be causing a gradual hypoxic to ischemic stress and this may induce the release of other factors such as excitotoxic amino acids which depending upon the duration could worsen the neurological outcome.

Effect of $+G_z$ Onset Rates on G-LOC Induction Time and Brain Metabolites

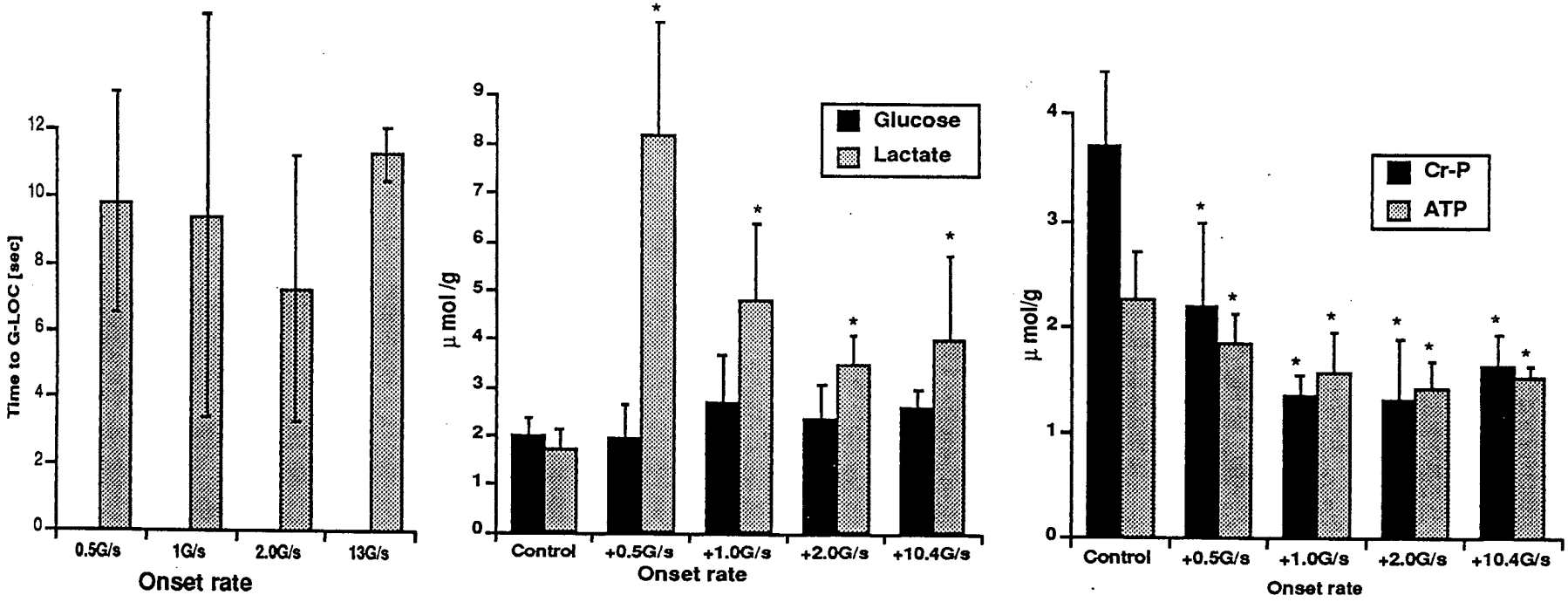


Figure 1: Mean ± SD (n=6/group) *P<0.05, as compared to control. Control rats were subjected to 2 exposures at 0.5Gz. The experimental rats were subjected to $+25G_z$ using the indicated onset rates.

SECTION 3

Determination Cerebral Blood Volume in Rats Exposed to +Gz (head -to-foot inertial force) in a Small Animal Centrifuge (SAC):

Measurement of CBF in brain is most difficult of any organ because of its vasculature. The common carotid, internal carotid, the vertebral and small spinal arteries supply blood to the brain via circle of Willis. These same arteries also supply blood to the scalp and noncerebral tissue. These factors limit the ability to measure the true CBF. Measurement of CBF during $+G_z$ stress presents even more practical problems. Due to these reasons determination of cerebral blood volume (CBV) is an attractive alternative. Since there a high degree of correlation between CBV and CBF determination of CBV should provide a good estimate of CBF. A technique generally used for measuring total hemoglobin (Hb) and iron content of the blood was modified for the brain tissue.

Centrifuge exposure protocol:

Protocol 1: Eight groups of rats (n=5, in each group) were exposed to a single + 0.5 to $25G_z$ for 30 second in the SAC and brain samples were collected by freeze fixation on liquid N_2 cooled plates as described previously while the centrifuge was running.

Protocol 2: Rats (n=5-6 at each time point) were subjected to a single +25 G_z exposure for 5 to 35 second and brain samples were taken as before. For the post centrifugation time points rats were exposed to +25 G_z for 30 second and brains were collected immediately, 15 and 60 second post deceleration. Control rats were exposed to +0.5 G_z for 30 second.

Brain perfusion: Halothane anesthetized rats (n=4) were perfused via aorta with 500 ml of saline containing 2 ml of heparin to remove blood and brains were removed, frozen in liquid N_2 and analyzed for iron content (from non blood sources).

Determination of total iron and hemoglobin (Hb): 100 mg of frozen brain tissue was homogenized in 1 ml of cold distilled water. For total iron determination 0.2 ml of concentrated HNO_3 was added to 0.1 ml of homogenate and boiled for 10 min in a boiling water bath. The mixture was centrifuge (10,000 rpm for 10 min) to remove any particulates and an aliquot of the supernatant was used for iron assay. A whole blood sample was collected from 6 rats and treated with HNO_3, centrifuged and an aliquot (0.08 ml) was used for the assay. The 2 ml assay mixture contained 0.08 ml of the sample 1.92 ml of 3 M sodium acetate and after the primary O.D. reading at 560nm, 0.015 ml of color reagent Ferrozone (Sigma kit # 565) was added and incubated for 10 min at 37 ^{0}C in a shaking water bath and the final O.D. reading was taken. Ferrozone gives a purple color at pH 4.5 (Stookey, 1970). Iron content was calculated and blood volume was calculated as follows:

$$\text{Blood volume (ml/g)} = \frac{\mu \text{ g iron /g brain tissue}}{\mu\text{g iron /ml blood}}$$

For total Hb determination to 0.1 ml of above tissue homogenate or blood, 1.9 ml of Drabkins reagent (Sigma kit #525) was added, incubated for 15 min and OD was taken at 560 nm. Total Hb was calculated using Hb standard and CBV was determined using the above formula.

Results:

Effect of $+G_z$ stress and duration on rat brain hemoglobin and Iron content and CBV: The data in Table 1 show that as the $+G_z$ level increased from 0.5 to 25 G, total Hb and iron content decreased significantly as compared to +0.5 G_z (control). This decrease was some what stablized at higher than +15 G_z.

Table 1

Effect of $+G_z$ Stress on Total Hemoglobin and Iron Content in Rat Brain

$+G_z$ Level	Hb (mg /g)	Iron (μg/g)
0.5	76.20 ± 5	231.46 ± 16
+ 10.0	57.57 ± 5.3 *	123.15 ± 12 *
+ 15.0	39.67 ± 5.0 *	93.40 ± 8.4 *
+ 17.5	35.25 ± 2.3 *	67.35 ± 6.7 *
+ 20.0	41.70 ± 2.8 *	94.09 ± 1.7 *
+ 22.5	41.78 ± 2.0 *	80.70 ± 2.3 *
+ 25.0	40.90 ± 2.0 *	62.61 ± 2.0 *

Mean ± SEM (n=5 in each group). * Significant.ly different from controls (0.5 Gz). Rats were exposed to a 30 sec exposure at the indicated $+G_z$ level and brain samples were collected immediately by freeze fixation as described under methods.

The time course (centrifuge Protocol 2) of changes in CBV is shown in Fig. 1a, ab and 1c. The highest decrease in Hb and iron content and CBV was observed 5 s after the onset of $+25G_z$. Centrifugation of higher duration did not show further significant decreases in either Hb or iron. However, immediately post-deceleration a significant increase in both Hb and iron content was observed. The post-centrifugation hyperemic response peaked at 15 s and returned to control levels within 60 s.

Conclusions:

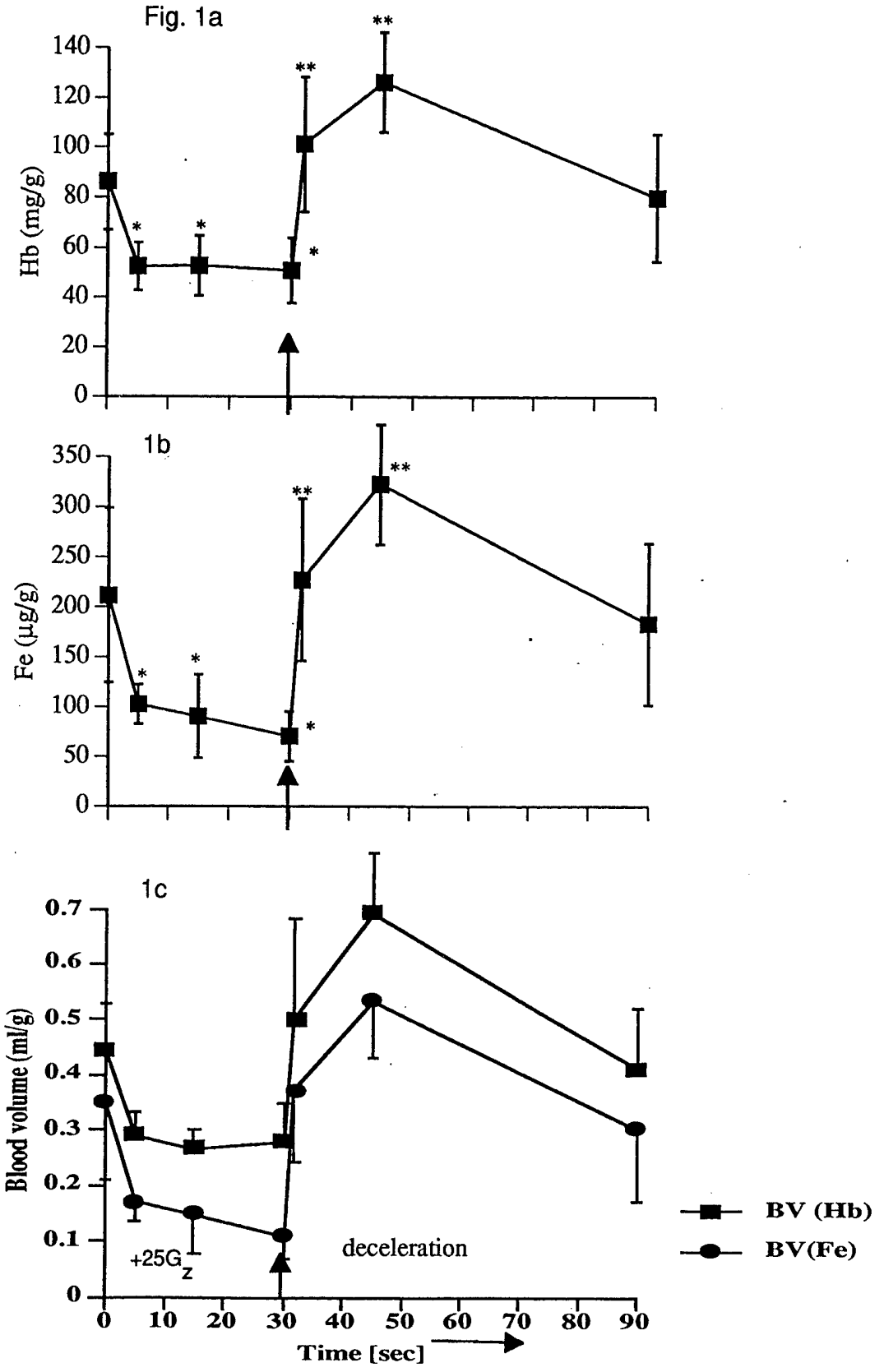
The results of this study clearly show that as the $+G_z$ stress (+10 to +25 G_z) was increased, total Hb, iron content and consequently CBV decreased significantly. This decrease stabilized or slightly increased as the $+G_z$ increased more than +17.5 G_z. Rats do not exhibit G-LOC at $+G_z$ level less than +20 G. Therefore it is likely that at lower $+G_z$ levels (< +17.5), CBF may not be completely stopped or reduced to a critical levels to cause G-LOC. But at higher $+G_z$ the reduction in CBF becomes critical to a point where the supply of energy is not sufficient to support neuronal function. This could cause G-LOC.

The largest decrease in CBV was observed within 5 second after the onset of $+25G_z$, with no or little further change as the duration increased. This is to be expected because the decrease in carotid artery blood pressure and presumably CBF drops to near zero within 2 s of $+G_z$ onset.

Therefore centrifugation of logner duration will not reduce CBF any further.

The data show that the decrease in CBV is between 40 to 50%. In other studies we have found that during +25G_z exposure CBF is reduced more than 99% within 2 s. This obvious discrepancy between CBF and CBV reduction can occur due to trapping of blood in the capilleries during high G exposure. It is likely that due to the sudden very high G force capilleries may collapse "trapping " blood. This situation can explain the discrepance between CBF and CBV in the present experiments.

Effect of a Single + 25G_z Exposure on Rat Brain Hb and Fe Content



* P < 0.05, compared to 0 time ** P < 0.05, compared to 30 sec time

SECTION 4

Measurement of Catecholamines, Acetyl choline and Choline in rat Brain Following $+G_z$ Exposure

Pilots are exposed to high acceleration forces in the head-to-foot ($+G_z$) direction during aerial combat, that can result in G-LOC within seconds (2). The use of G-suits, straining maneuvers and centrifuge training have reduced the incidence of G-LOC, still it is a significant problem. We believe that an understanding of neuromechanism of G-LOC will provide a basis for pharmacological intervention to prolong the time to G-LOC. We have shown that in rats exposed to $+22.5G_z$, there is a complete cessation of CBF with in 2 s, an increase in adenosine and lactate in 5-10 s, and a decrease in glucose and ATP levels occurs within 15-30 s. Even though all these events take place at or near the time of G-LOC there causal relationship (to G-LOC) is unknown. Adenosine, released during ischemia, is an important cerebral vasodilator, inhibits the release of NTs, and plays a protective role against ischemic stress. NTs play an important role in maintaining cognitive function with their synthesis and release is closely tied to the availability of glucose and oxygen. It is hypothesized that their impaired synthesis/release is the neurochemical link between the $+G_z$-induced ischemia and G-LOC. In these studies level of NTs and catecholamines was measured in whole brain following $+22.5G_z$ exposure in normoglycemic and hyperglycemic rats. Moderate hyperglycemia (HG) was found to significantly increase the G-LOC induction time in rats (a manuscript of this work is described elsewhere in this report).

Neurotransmitters are synthesized, stored and released from the presynaptic nerve terminals. The arrival of a nerve impulse causes an influx of Na^+ and efflux of K^+. This generates an action potential that subsequently results in membrane depolarization. These events elicit Ca^{++} entry into the cell, triggering the release of NTs into the synaptic cleft where they bind to specific receptors on the post synaptic terminal. NTs either depolarize (excite) or hyperpolarize (inhibit) the nerve cell. Synaptic transmission is terminated by either the cessation of release, or by the inactivation of NTs. This entire process, synthesis and degradation requires a continuous supply of ATP and oxygen and thus is likely to be impaired during ischemia.

Ach is synthesized from choline and acetyl coenzyme A, that is produced by oxidative metabolism of glucose. During ischemia Ach synthesis is inhibited due to a lack of acetyl coenzyme A, due to a decrease in glucose and oxygen supply (7, 13). Ischemia (5 to 60 min) has been shown to decrease Ach levels transiently in many areas of the brain (7, 18). Ach is believed to be a key in cognitive function. An agonist for Ach release, 3-4 diminopyrdine, has been shown to improve hypoxia-induced behavioral deficits (4, 9). A decrease in Ach release during deep sleep has been reported (1). However, no work has been done to evaluate the role of compromised Ach release or turn over during the initial seconds following ischemia. This information could be of crucial importance in understanding the neurochemical basis of G-LOC.

Alterations in the level of DA (10,11) and monoamines (5, 6, 13) and their metabolites during and after cerebral ischemia ($\geq$ 5 min) have also been reported. Release of DA and 5-HT from the striatum increased greatly when CBF was reduced to < 20 % of normal, a level that causes I-EEG (11, 19). DA is also the precursor of NTs norepinephrine (NE) and epinephrine (E)

whose levels are also altered during ischemia (7). The cell bodies of dopaminergic neurons are located primarily in the mid brain. DA acts as a vasoactive agent via specific receptors on vascular smooth muscles. In addition, DA is involved in conscious perception, emotions and memory (10,11). Serotonin or 5-hydroxytryptophan (5-HT) is found in the brain stem, the pons and the raphe nuclei. It is also a vasoactive agent that exerts control over cerebral circulation and is believed to be involved in sleep regulation and in the neural basis of consciousness (6, 8).

Our working **hypothesis** is that, *following a sudden high +Gz -induced cessation of CBF and anaerobic glycolysis, there is an increase in the vasodilator adenosine that modulates the NT's release or turnover in the brain such as the corticothalamic or reticular formation signalling the loss of neuronal function or G-LOC to conserve energy.* Adenosine also inhibits the release of excitotoxic NTs like glutamate. The excitotoxic amino acids (glutamate) are released during ischemia of longer duration and are implicated in ischemic pathology. This proposal is only concerned with very early and acute events of very short duration (30 s) ischemia, that are not likely to result in pathology, thus our studies will not be focused on glutamate.

+G_z exposure protocol : All hyperglycemic groups (HG) received two exposures at +22.5 G_z, 30 min apart. During the first exposure, EEG was recorded during and after centrifugation. During the second +22.5G_z exposure, the centrifuge run was terminated at the precise moment when EEG became isoelectric (I-EEG). The brains were collected 30 s post-centrifugation by freeze fixation. The brains were pulverized and stored at -70^{0}C until analyzed. In separate experiments different groups of rats treated exactly as above but the brain samples were collected at 30 s during centrifugation.

One saline injected group received two exposures of +0.5G_z, 30 s each and brain samples were obtained 30 s after deceleration by freeze fixation. The other saline injected group was exposed to two exposures of +22.5G_z, and the brains were collected exactly as in HG rats.

Measurement of Catecholamines: 50 mg of frozen brain tissue was homogenized in 0.5 ml of mobile phase and centrifuged for 10 min at 10,000 rpm. The supernatant was filtered(0.45µ filter) and 50 µl was used for HPLC analysis. Catecholamines were separated on a 15 cm C18 Bechman column electrochemical detection at a flow rate of 1 ml /min (2). The mobile phase consisted of citrate buffer pH 4.3 containing 50 mM citric acid, 100 mM sodium acetate, 0.17 mM EDTA, 0.34 mM sodium octa sulphonate and 8% methanol.

Measurement of acetyl choline (ach) /choline (ch): Frozen brain tissue (50 mg) is homogenized (1:10) in 7% cold percholric acid and centrifuged at 10,000 rpm for 10 min. A 20 µl aliquot of the supernatant is analyzed as follows. These compounds were measured using enzyme conjugated column and analytical column kit from Bioanalytical Systems of Lafayette, IN. A polymer analytical column is connected to a column conjugated with acetyl choline esterase and choline esterase column. The mobile phase consisted of 50 mM sodium phosphate buffer containing 1% antimicrobial agent Kathon. Ach is hydrolyzed into choline and acetate which in turn by choline esterase action are oxidized into hydrogen peroxide and betaine. Hydrogen peroxide is then detected by electrochemically on a platinum electrode.

Results and conclusions:

The data show that a 30 s exposure of normoglycemic (NG) rats to +22.5G_z had no significant effect on dopamine (DA) or its metabolite DOPAC and HVA (Fig. 1a). In addition, no significant differences were observed in the level of 5-hydroxy tryptamine (5-HT) or its metabolite %-hydroxyindole acetic acid (5-HIAA) and the level of norepinephrine (NE) (Fig. 1b and 1c). However the level of NE was slightly higher in both normoglycemic or hyperglycemic (HG) rats exposed to +22.5G_z as compared to control rats.

The level of Ach was significantly reduced in NG rats exposed to +22.5G_z for 30 s. Rats injected with 0.625 mg/kg glucose the Ach levels were also reduced. As the level of HG was increased from 1.25 to 7.5 mg /kg the level of Ach appears to increase (Table 1). In other studies ischemia of longer than 5 min duration decreases the concentration of catecholamines and Ach with a corresponding increase in ch (3,13,7). Our preliminary data for the first time shows differences in the neurotransmitter Ach within 30 s of ischemia. This could result from either increased breakdown or reduced synthesis of Ach. Ach is synthesized from acetyl coenzyme A and choline. It is proposed that due to +Gz induced ischemia glucose is metabolized by anaerobic glycolysis. As a consequence pyruvate is converted into lactate and not in acetyl CoA. This could result in a reduction in the level of Ach. Furthermore it is speculated that cholinergic system may play some

Protocol	Acetyl choline (nmol/g)	Choline (nmol/g)
Saline		
+0.5G_z (30s)	4.24 ± 2.7	38.37 ± 13
+22.5G_z (30 s)	0.98 ± 1*	43.02 ± 15
HG (mg glu /kg)		
+22.5 G_z (30s)		
0.625	0.60 ± .01	43.50 ± 15
1.25	4.34 ± 3.5	49.57 ± 17
2.5	5.80 ± 3.7	42.92 ± 16
5.0	7.24 ± 0.7	39.90 ± 11

in causing G-LOC. These studies are preliminary and further investigation are needed for confirmation.

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Figure 1 Brain Catecholamine levels following 30s exposure at +22.5Gz in normo and hyperglycemic rats

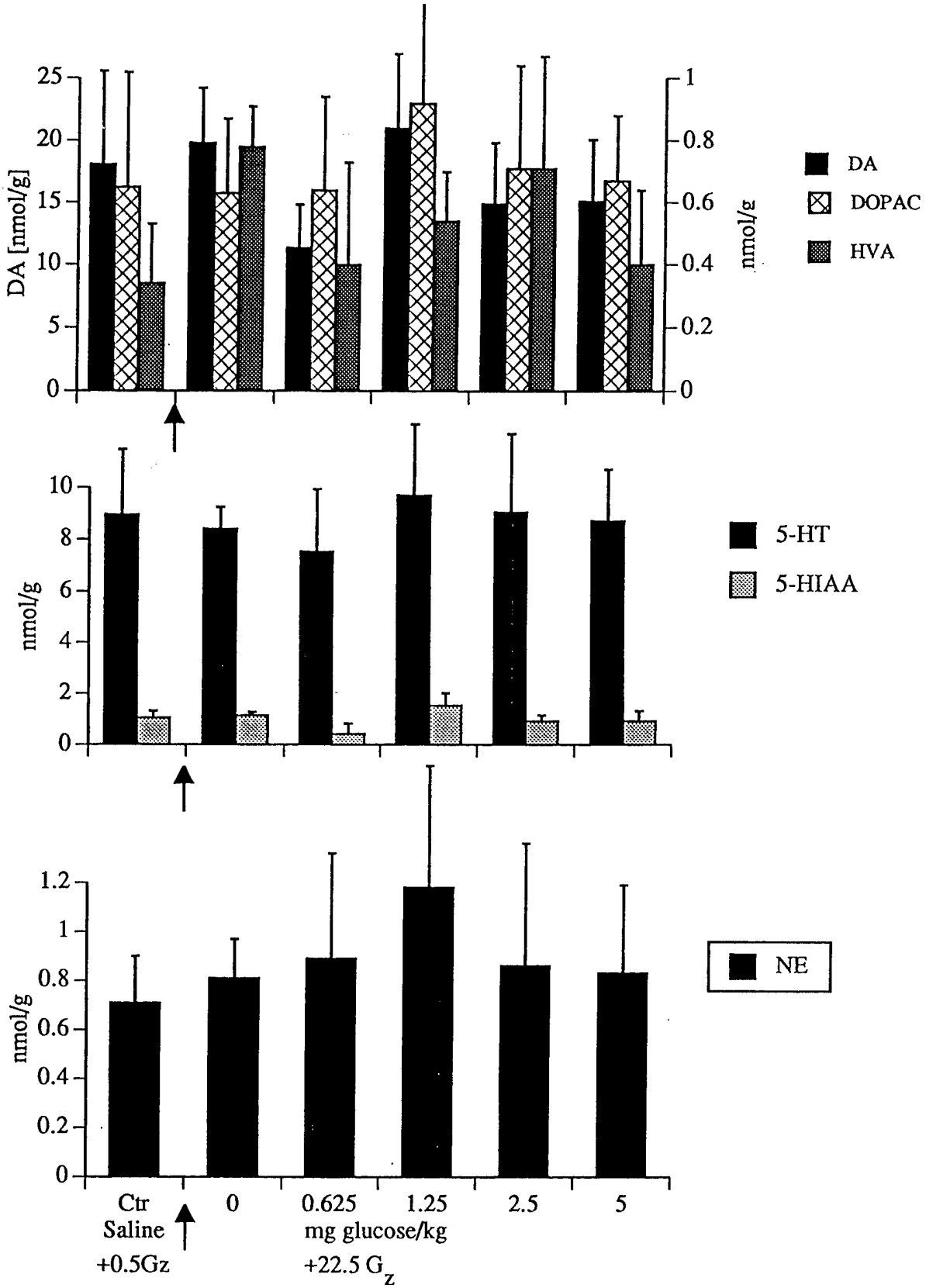
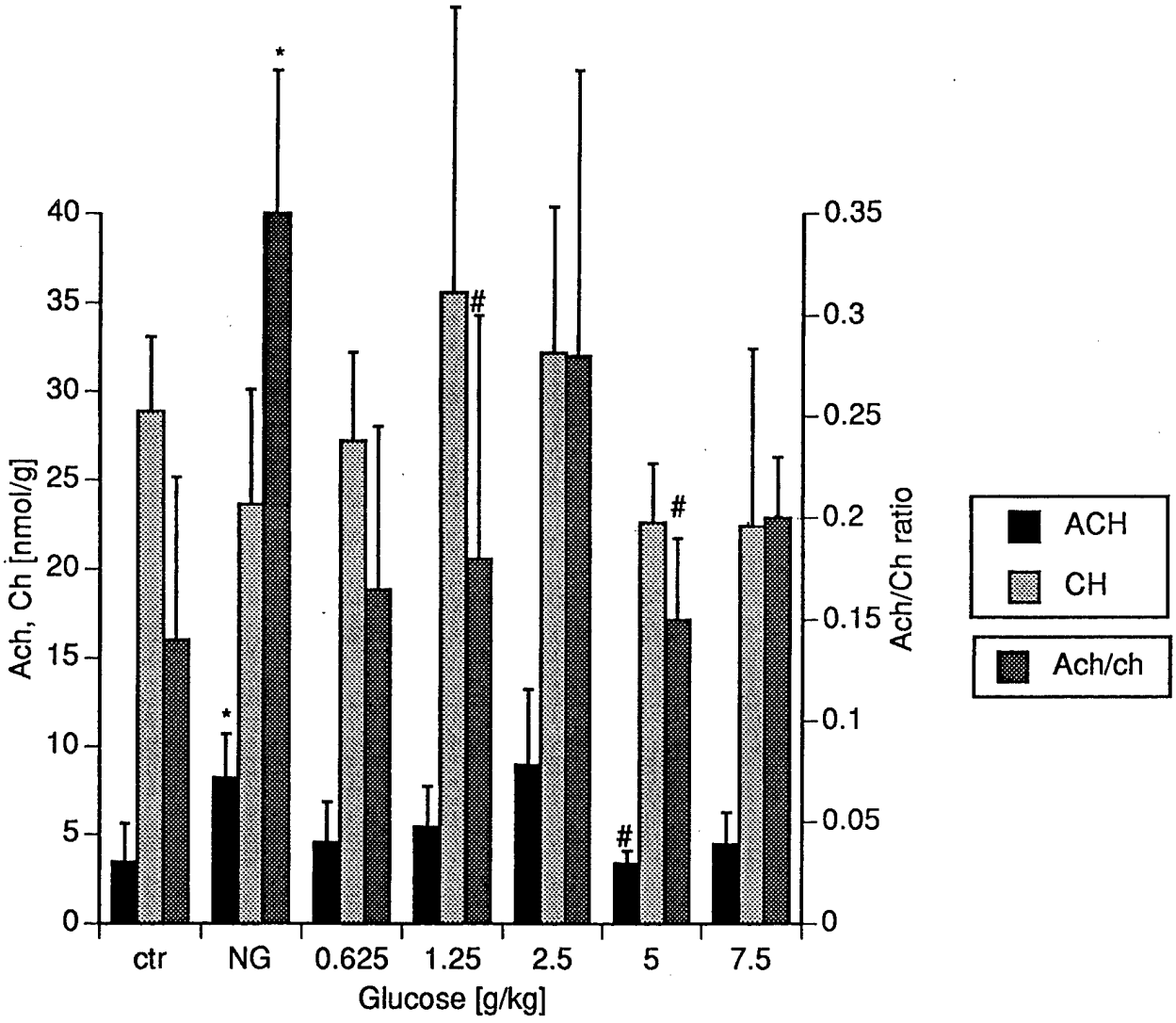


Figure 2: Brain acetylcholine and choline levels in normo and hyperglycemic rats during exposure to +22.5G_z exposure



* P < significantly different than control.
# P < significantly different than NG

SECTION 5

Moderate Hyperglycemia Increases G-LOC-Induction Time in Rats

Introduction:

Pilots of high performance aircraft are exposed to hypergravic stress which can cause loss of consciousness (G-LOC). Many factors such as heat stress, hypoglycemia, missed meals or insufficient caloric intake (Balldin, 1984, Lyons et al. 1992) hyperventilation, hypoxia, hypocapnia, dehydration, and height of the individual (Gillingham et al. 1986) etc. effect G-tolerance negatively. In the last several decades, human centrifuges have been used to investigate complex physiology imposed by the high $+G_z$ environment. This research has resulted in the development of the anti-G straining maneuver and the anti-G suit to increase $+G_z$-tolerance and to reduce G-LOC incidents. However, G-LOC remains a source of great concern for the Air Force and the aeromedical community.

We believe that a complete understanding of the neurophysiologic and neurometabolic ramifications of high $+G_z$ exposure could result in useful strategies to prevent or reduce the incidence of G-LOC. For this purpose, a rodent-centrifuge model has been developed at the Armstrong Laboratory, Brooks AFB (Werchan and Shahed, 1992). This pioneering research has shown that brief $+G_z$ exposures of rodents in a small animal centrifuge (SAC), depending upon the level results in partial to complete reduction in cerebral blood flow (CBF) or global cerebral ischemia (Werchan and Shahed 1992, Shahed et al., 1994, Shahed et al.*in press*). This results in a loss of glucose and oxygen supply and consequently in the energy production in the brain. It was proposed that G-LOC or loss of neuronal function occurs to reduce energy consumption. Since neuronal function accounts for more than 50% of total energy consumption of the brain (Erecinska and Silver, 1989). Since glucose is the primary substrate for energy (ATP) production, we hypothesized, that if its supply of the brain is potentiated, onset of G-LOC during high $+G_z$ exposures could be delayed.

In the present study the effect of moderate hyperglycemia on the onset of G-LOC during $+G_z$ exposures of the conscious rats in the SAC was investigated.

Methods:

EEG electrode implantation and $+G_z$ exposure in the SAC: Male Sprague-Dawley adult rats (250 to 400 g) were provided free access to food and water. EEG electrodes were implanted surgically under Halothane anesthesia as previously described in section 1.

Hyperglycemia (HG) was induced in five groups of rats (n=5/group) by intraperitoneal (ip) injection of 50% glucose solution to provide final concentration of, 0.625, 1.25, 2.5, 5.0 and 7.5 g glucose/kg body weight. Rats were injected 30 min prior to centrifugation. Two groups of

control rats were similarly injected with saline.

+G_z exposure protocol : An onset/offset rate of 10.4 +G_z/ sec was used in all protocols. All hyperglycemic groups (HG) received two exposures at +22.5 G_z, 30 min apart. During the first exposure, EEG was recorded during and after centrifugation. During the second +22.5G_z exposure, the centrifuge run was terminated at the precise moment when EEG became isoelectric (I-EEG). The brain was collected 30 s post-centrifugation by freeze fixation (Shahed et al., 1994). The brains were pulverized and stored at -70^{0}C until analyzed.

One saline injected group received two exposures of +0.5G_z, 30 s each and brain samples were obtained 30 s after deceleration by freeze fixation. The other saline injected group was exposed to two exposures of +22.5G_z, and the brains were collected exactly as in HG rats.

For the measurement of blood gases and glucose a small blood sample (0.5 ml) was obtained before, and 15, 30, 60, 3 hr and 24 hr after the ip injection of different doses of glucose.

Assay of Metabolites: Frozen brain tissue (0.2 g) was homogenized in 2.0 ml of 7% cold perchloric acid, and an aliquot of the neutralized supernatant was used for the determination of metabolites. The level of glucose and lactate were measured spectrophotometrically and creatine phosphate (Cr-P) and ATP concentration were determined by high pressure liquid chromatography as previously described (Shahed et al. 1994).

RESULTS:

Effect of hyperglycemia on blood chemistry: Blood glucose levels peaked within 30 min and remained at this elevated level for 3 hr and returned to basal levels 24 hrs after the ip injection of glucose. Table 1 shows that blood glucose levels increased (1.36 to 3.66 fold) as the dose of glucose injection varied from 0.625 to 7.5 g glucose /kg. No significant differences in blood lactate, pH, pCO_2 , pO_2 or hematocrit were observed between HG and NG rats over a 24 hr period (data not shown).

Effect of HG on G-LOC induction times and metabolites: Rats were exposed to +22.5G_z and EEG was recorded until G-LOC (isoelectric EEG or I-EEG) was evident. In NG rats G-LOC occurred at 13.25 ± 2 s (Figure 1a) as shown previously (Werchan and Shahed, 1992). The G-LOC induction times during the first and second +22.5G_z exposures were similar during the first and the second +G_z exposure and thus the mean times are shown in Table 1. The G-LOC induction times in the HG rats increased by 2.65, 5.15 ($p<0.05$), 5 ($P<0.05$), 2.44 and 1.76 fold as the dose of glucose injection increased from 0.625 to 7.5 g/kg respectively (Table 1).The recovery times in the HG (22 to 40 s) groups were higher than the NG (17.4 ± 5 s) group they were not statistically different.

For metabolic analysis brains were collected 30 s post-centrifugation (reperfusion). The level of lactate in the NG and all the HG groups was significantly higher than the control group exposed to +0.5G_z (Fig. 1b). Lactate levels in the 1.25 and 2.5 g HG groups were significantly different than the NG exposed to +22.5G_z . Brain glucose levels in the 5.0 and 7.5 g HG groups

were significantly higher than the NG rats (Figure 1b). The level of creatine phosphate was significantly lower in all groups (except 0.625 g HG) than the control group (Figure 1c). ATP levels, although lower were not statistically different than the control (Figure 1c).

Discussion:

The magnitude of $+G_z$ exposure, duration, reduction in cerebral blood flow, and the onset rate are among the major factors which contribute to G-LOC in the pilots. An analysis of 18 G-LOC mishaps revealed missed meals as a possible contributing factor in 7 incidents (Lyons et al. 1992). In an earlier study (Britton, Corey, Stewart; 1946) reported a significant increase in blood glucose concentration in proportion to the magnitude of $+G_z$ exposure in cats. These studies suggest that systemic glucose (primary energy substrate in the brain) levels may be important in G-tolerance. The results of the present study for the first time show that moderate HG significantly increases the G-LOC-induction time compared to NG rats during $+G_z$ exposure (Table 2, Fig.1a). The glucose response was biphasic and shows a bell shaped curve (Fig.1a). These data show that an optimum blood glucose level may be needed to increase G-LOC induction time.

During high $+G_z$ exposure blood flow to the brain ceases or critically reduced (ischemia), consequently depriving the brain of glucose and oxygen required for energy (ATP) production. Neuronal function is dependent upon an uninterrupted supply of ATP. Thus we hypothesized that increasing blood glucose levels before $+G_z$ exposure would also increase brain glucose level which, in turn, could boost brain energy levels. This hypothesis is supported by the data in Fig. 1b and 1c. Brain glucose levels were higher than the saline control in all HG groups except the 1.25 and 2.5 glucose groups. The latter two groups also showed a significant increase in the G-LOC-induction (over 5 fold) time over the NG rats during $+22.5G_z$ exposure. Therefore, it is suggested that in the 1.25 and 2.5 g/kg glucose group the rat remains conscious for a longer time and utilizes glucose as proven by the highest lactate production. Consequently the brain glucose levels were not significantly higher than the saline injected group. Similarly in the 5.0 and 7.5 g.kg glucose group brain glucose levels were significantly higher because the G-LOC induction time not significantly different than the saline group. Since the G-LOC occurs sooner, the metabolic demand is also reduced and glucose is not utilized. In addition, the level of Cr-P and ATP were not significantly different than the NG control even though the rat was conscious for a longer duration. This suggests that the level of Cr-P and ATP were maintained longer in the HG than in the NG group. In non-centrifuge models of pre-ischemia, HG has been shown to delay ischemic depolarization, and lower the rate of ATP depletion (Ekholm et al., 1993, Hsu et al., 1994). It is proposed that moderate HG during short duration ischemia prolongs the onset of G-LOC by maintaining the level of higher energy phosphates.

Pre-ischemic HG, due to lactoacidosis and hyperosmolality (causes edema) worsens the post-ischemic neurological outcome (Klatzo, 1967). In contrast, others have reported absence of increased postischemic edema in HG rats (Gisselsson et . al., 1992) and increase in brain ATP concentration without lowering intracellular pH (Hsu et al., 1994). In most studies where preischemic HG resulted in worsened neurological outcome ischemic duration was much longer than the 15 to 70 s in the present study. Also the brain lactate levels (6-7 μmol/g) observed in this study were much lower than those associated delayed ischemic damage.

In summary, the results show that moderate HG increases the G-LOC induction time during $+G_z$ exposure. These results could be of important significance in pilots exposed to $+G_z$ stress in high performance aircraft.

Table 1

Blood glucose concentrations

Protocol	Control	30 min post Inj.	Fold increase
Glucose [g/kg]			
0.625	110.6	150.1	1.36
1.25	167.2	275.4	1.65
2.5	164.7	270.1	1.65
5.0	191.8	700.6	3.65
7.5	179.8	658.3	3.66

Representative data from one rat in each group. A small blood sample was drawn before and one 30 min after intraperitoneal injection of glucose. Values are mg glucose /DL.

Table 2

Effect of Hyperglycemia on G-LOC and Recovery Times

Protocol	G-LOC induction time [seconds]	Recovery time [seconds]
Normoglycemic	13.25 ± 2	17.40 ± 5
Hyperglycemic (g/kg)		
0.625	35.20 ± 15	38.40 ± 14
1.25	68.25 ± 50*	42.40 ± 24
2.50	65.80 ± 35*	28.70 ± 17
5.00	32.40 ± 14	40.80 ± 14
7.50	23.42 ± 10	22.17 ± 11

Mean ± SD (n=6/group) * $P < 0.05$, as compared to $+22.5G_z$ (NG) group. Thirty minutes after ip injections of glucose, rats were subjected to $+22.5G_z$ until I-EEG or G-LOC was evident and the centrifuge run was terminated. EEG was monitored during recovery until normal EEG was visible.

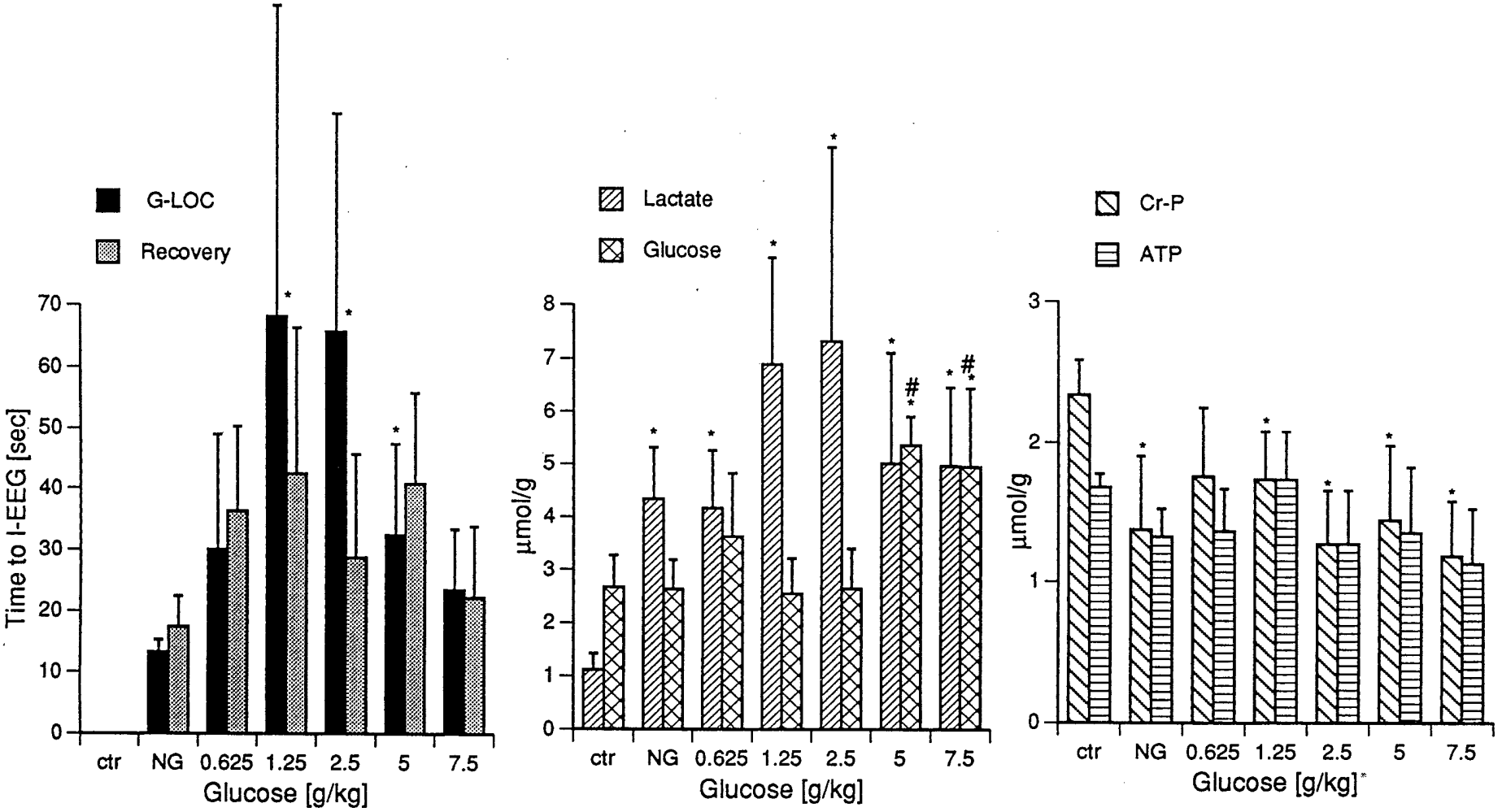


Figure 1. Effect of hyperglycemia on G-LOC induction/recovery times and glucose and energy metabolites in rat brain.

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SECTION 6

High Acceleration Induces Alterations in Cerebral Blood Volume and Metabolism in Mice

P. M. Werchan and A. R. Shahed. Armstrong Laboratory Crew Technology Division and Operational Technologies Corp.

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ABSTRACT:

Acceleration stress in the head-to-foot vector ($+G_z$), depending upon the level, can cause ischemia with subsequent loss of consciousness (G-LOC) in both humans and animals. It has been hypothesized that $+G_z$-induced reductions in CBF may show a gradient from the regions distal to those proximal to the heart. To test this hypothesis, we measured CBV and metabolic alterations in mice brain before, during and after $+G_z$ exposure in a small animal centrifuge (SAC). This unique SAC allows brain collection by microwave fixation (MWF) during and post-centrifugation in < 1s. Complete global ischemia can be induced within 2 s. Mice subjected to various $+G_z$ levels exhibited G-LOC (isoelectric EEG) within 14.2 ± 3 s during a $+32.5G_z$ exposure. A single, 30 s $+G_z$ (10 to 35) exposure, resulted in a significant increase in lactate and a decrease in creatine phosphate (cr-P) levels beginning at $+15G_z$ level. A decline in CBV and ATP was observed as the $+G_z$ level increased, and became significant at +25 and $35G_z$. The time course of changes in CBV and metabolites in the whole brain and various regions of the brain were measured during a single $+35G_z$ exposure. The level of Cr-P and ATP decreased ($p < 0.05$) and the lactate increased within 15-35 s of exposure. Lactate accumulation was highest in the hippocampus (HIP) and the cortex (CTX). CBV decreased in all regions, but was highest in the CTX and the HIP. Post-centrifugation hyperemia was observed in all regions except the CTX and HIP. This lack of hyperemia in the CTX could be due to hydrostatic column effect (most distal to the heart) and may delay lactate clearance resulting in higher accumulation. It is concluded that $+G_z$ exposure depending on the level causes partial to complete global cerebral ischemia. However, due to the observations in the CTX and HIP, a hydrostatic column effect during $+G_z$ exposure can not be ruled out. The results also show that the noninvasive rodent-SAC model could be ideal for studing short term ischemia and reperfusion.

Introduction:

High $+G_z$ exposure in pilots of high performance aircraft remains a significant concern of the Air Force and aeromedical community due to the possible incidence of G-LOC. Pilots flying a high performance aircraft in aerial combat are exposed to high acceleration forces in the head-to-foot direction. This (centripetal acceleration) generates an inertial reaction of equal but opposite force (G) which displaces organs and forces blood towards the lower extremities. This reduction in cerebral blood flow (CBF) is a primary cause for G-LOC. The existing bench top models of cerebral ischemia are inadequate to assess accleration stress on the brain. We, therefore developed a unique small animal centrifuge (SAC). In the SAC, rodents (rats and mice) can be exposed to various $+G_z$ levels and many physiological parameters (EEG, ECG, CBF and intracranial

pressure, systemic blood pressure) can be simultaneously monitored. In addition, the brains can be obtained at any time point during or after centrifugation for biochemical analysis (25). Previously, we found that a 30 s exposure of rats to $> +20G_z$ in the SAC caused a cessation of carotid artery blood flow (24), a reduction in brain glucose, Cr-P, and ATP, an increase in lactate production, and an isoelectric EEG (G-LOC) within 15 s (18). These metabolic alterations are consistent with the acute stages of global cerebral ischemia.

The brain is heterogenous in its cellular makeup and function and may also show cellular metabolic compartmentation (27). Differences in regional CBF and glucose utilization during ischemia in rodents have been previously reported (5, 13). In the studies of drug induced coma, a decrease in the concentration of high energy phosphates (without an increase in lactate production) was observed in the reticular formation but not in the posterior colliculus of mice (10). They suggested that a decrease in the reticular formation energy stores could be a signal to reduce brain activity (coma) to conserve energy (10, 11). Measurement of CBF in baboons during high $+G_z$ exposure was reported to be nonuniform and showed regional variability (23). Based on these studies, we sought to investigate whether $+G_z$-induced ischemia is global or shows regional preferences.

To investigate the effect of $+G_z$ exposure on different regions of the brain, a tissue fixation device was needed that could inactivate the brain enzymes quickly during centrifugation while preserving structural integrity. MWF has successfully satisfied both criterias (11, 12, 21). In order to fix brains during and after centrifugation the MWF device was modified to rotate with the centrifuge. In the present study, we investigated the effect of acceleration stress on cerebral blood volume (CBV), glucose and energy metabolism in the whole and in different regions of mice brain. Due to a high degree of correlation between CBF and CBV (16) and the practical difficulties in measuring CBF during centrifugation, we measured CBV by determining the total iron content of the brain (14).

MATERIAL AND METHODS:

Animals: Adult male mice (BALB/cAnNCrlBR 30-40 g, Charles River Laboratories, Wilmington, MA) were provided free access to food and water.

Small Animal Centrifuge (SAC): The SAC was specifically built to expose rodents to $+G_z$ and has been previously described (18, 19, 25). The operation of the SAC is fully computerized. Eight channels of physiological data can be collected simultaneously and stored on optical disc drives for subsequent analysis. A modified 2450 MHz, 10 KW microwave heat inactivation system focuses microwaves directly on the mice brain via a waveguide which rotates with the centrifuge. Fully awake mice were placed individually in a Plexiglas cylindrical holder with the teeth anchored on a bite bar. The hind portion of the mouse was cushioned with dense foam pads to prevent stretching during centrifugation. The MWF system delivers heat directly to the mouse head sufficient to inactivate the brain enzymes in < 600 ms. The MWF system can be activated via a computer anytime during or after centrifugation.

EEG electrode implantation and monitoring: Under halothane anesthesia, biparietal lateral EEG electrodes were implanted on the mouse cranium using two #000-120 x 1/16" stainless steel screws and washers (#000). A third screw (ground) was placed slightly cranial to the bregma suture and lateral to the midsagittal suture. All screws and attached wires were imbedded in cranial plastic cement and the wound site closed. After fully awake (two to three hours), the mouse was placed in a Plexiglas holder and horizontally affixed to the SAC arm with the head facing the center

shaft of the centrifuge (+G_z orientation). Electrodes were attached to amplifiers for monitoring EEG during and following +G_z exposure. Electrode implanted mice were not used for MW fixation. G-LOC was defined as the appearance of I-EEG (3, 25).

+G_z exposure protocols: In all experiments an onset rate of +10.4 G_z/s was used. Control mice were exposed to +0.5 G_z for 30 s (unless stated otherwise) to incorporate stresses imposed by restraint, rotation, and noise. Brain samples were collected by MWF (power setting 3.7, 600 ms) at 30 s for controls and at indicated times for experimental protocols. Brains were removed, weighed, pulverized in liquid N_2, and stored at -70°C.

Protocol 1: Determination of +G_z tolerance of mice: Fully awake mice (n=10) with implanted EEG electrodes were subjected sequentially to 30 s exposures each of +0.5 (control), 10, 15, 20, 25, 30, 35 or 40 G_z. A 5 min recovery period was allowed between each run and EEG was monitored and stored on optical discs for subsequent analysis.

Protocol 2: Effect of increasing +G_z levels on metabolites and total iron content (CBV) in whole brain: Seven groups (n=6/group) of mice (without electrodes) were subjected to a single 30 s exposure of either +0.5 (control) or +10 to 35 G_z (2.5 G increment) and brains were microwaved during centrifugation.

Protocol 3a: Time course of metabolic changes in the whole brain during a single exposure at +35G_z: Four groups (n=6/group) of mice were subjected to +35 G_z for either 5, 15, 25 or 35 s and brains were microwaved at these time points. Four additional groups of mice were subjected to +35G_z for 35 s and brain samples were collected 2, 15, 30 and 60 s post-centrifugation.

Protocol 3b: Time course of alterations in regional CBV and metabolites: The centrifuge protocol was exactly the same as protocol 3a. Microwaved whole brains were dissected on ice into the cortex (CTX), cerebellum (CERE), mid brain (MB), brain stem (BS) and hippocampus (HIP). Brain tissue was used for metabolic analysis. Three groups of mice (n=6/group) were exposed to +35G_z for 30 s and brains microwaved at 30 s (during centrifugation) or 30 and 60 s after the run. The control group was exposed to +0.5G_z for 30 s and brains were similarly dissected. For the measurement of CBV, three groups mice (n=5 /group) were exposed to +35G_z for 30 s and brains were microwaved at 30 s (during centrifugation) and 30 and 60 s post-centrifugation. The brains were dissected as above and used for measuring total iron content (CBV).

Assay of Metabolites: Microwaved brain tissue was homogenized in 7% cold perchloric acid (1:10) using a Polytron homogenizer and centrifuged for 10 min at 10,000 rpm. Lactate and glucose were measured in the supernatant as previously described (18, 19). An aliquot (100 to 200 µl) of the supernatant was filtered (0.45 µm), and creatine phosphate (Cr-P), and adenine nucleotides were measured by ion pair reversed phase HPLC on a Waters NOVA PAK C_{18} column (25). Protein was measured using a BCA Protein Assay Kit (Pierce, Rockford Ill). Total iron content and CBV was determined as described before (19).

Statistical analysis: The data were analyzed by one-way analysis of variance (ANOVA) and Dunnett's follow-up test for multiple comparisons. All data are presented as means ± SD of 5 or 6 animals.

RESULTS:

Determination of $+G_z$ tolerance of mice: (Protocol 1): Mice exposed to different $+G_z$ levels for 30 s each, demonstrated a decrease in EEG amplitude at higher than $+25G_z$ (Fig. 1). The average $+G_z$ tolerance (G level at which I-EEG occurred) of mice was found to be 32.5 ± 7 under these conditions. The EEG became isoelectric (G-LOC), on average, at 14.2 ± 3.7 s after the onset of $+G_z$ and returned to normal between 18.1 ± 5 s post-centrifugation.

Effect of increasing $+G_z$ levels on metabolites and total iron content (CBV) in whole brain (Protocol 2): The data in Fig.2a show that a single 30 s, exposure of either +10 to 35 G_z caused a significant increase in lactate (2.2 to 3.3 fold) and a decrease in Cr-P (31-61%). A slight decrease in ATP levels was evident at low $+G_z$ levels that became statistically significant at $> +25G_z$. Glucose levels decreased significantly only during the $+35G_z$ exposure (Fig. 2a). The level of AMP and Adenosine also increased as the level of $+G_z$ exposure was increased (Fig. 2b). A progressive decrease in CBV was observed as the $+G_z$ levels increased. A statiscally significant decrease was observed at and higher than $+20G_z$ (Fig.2a).

Time course of metabolic changes in the whole brain during a single exposure at $+35G_z$ (Protocol 3a): A significant decrease in glucose, and an increase in lactate production were observed within 15 s after the onset of $+35G_z$ (Fig. 3a). The concentration of Cr-P decreased significantly within 5 s after the $+G_z$ onset but a significant decrease in ATP was not evident until 35 s. The concentration of AMP increased ($P< 0.05$), starting at 25 s during exposure, and continued to be elevated (> 3 fold) 60 s post-centrifugation. Adenosine concentration increased significantly during the $+G_z$ exposure but decreased sharply at the time of the termination of the centrifuge run and presumably resumption of CBF (Fig. 3b).

Time course of alterations in regional CBV and metabolites: (Protocol 3b): Microwaved brains were dissected into the CTX, CERE, MB, BS and HIP after exposure to $+35G_z$ for 30 s. The CBV decreased in all regions (Fig. 4) with the largest decreases observed in the HIP (75%) the CTX (50%), BS (50%), MB and the cerebellum (22-to-25%). All regions, except the CTX and the HIP, showed significant hyperemia 30 s post-centrifugation. The CBV returned to control levels in all regions except the HIP (significantly lower than control) and the CTX, 60 s post-centrifugation.

To determine the time course of regional metabolites different groups of mice were exposed to protocol 3b and the results are shown in Fig. 5. Glucose levels decreased ($\approx$ 30%) during 30 s of centrifugation and remained lower than the control until 60 s post-centrifugation. Lactate levels increased significantly in all regions at the 30 s time point during centrifugation, but was highest in the CTX (423%) and the HIP (353%) 30 s post-centrifugation. The concentration of Cr-P decreased significantly in the HIP, CERE (32%), BS (50%), CTX (53%) and the MB (67%) during centrifugation. Recovery of Cr-P required more than 60 s in all regions except the HIP and CERE. The basal levels of ATP and Cr-P were different and in general lower than CTX in other regions. ATP levels decreased significantly in the CTX (26%), the BS (44%) and the HIP (15%) but remained unchanged in the CERE and MB during the 30 s centrifugation. The recovery of all metabolites to control levels required 5 min post-centrifugation.

Discussion:

The relative $+G_z$- tolerance (G-LOC occurence) of humans to rodents is determined by the eye-to-heart distance. The $+G_z$ tolerance of mice was found to be 32.5 ± 7, which is higher than the $+22.5$ G_z level determined for rats and $+ 5.4 \pm 0.9$ G_z for humans (4). When mice were exposed to high $+G_z$ in the SAC, G-LOC (I-EEG) was evident at 14.2 ± 3.7 s after the onset of $> +30G_z$. Full EEG recovery (to pre-centrifugation) occurred, on the average, at 18.3 ± 5 s post-centrifugation. Loss of EEG has been associated with complete global ischemia in a non-centrifuge model, where CBF was halted by vessel occlusion (1). Spectral analysis of our rat EEG data revealed a significant reduction in total EEG power and predominance of delta frequency during prior to the G-LOC period (data not shown). Delta waves are known to predominate prior to unconsciousness and have been previously reported in other animals during acceleration stress (2).

In this study, regional and whole brain metabolic changes were measured for the first time using a MWF device during centrifugation. MWF was found to be effective during centrifugation since no or little enzyme activity was detected in microwaved mouse brains (not shown). Also, the baseline level of metabolites in the present study were similar to those previously reported (21). The degree of metabolic changes were qualitatively proportional to the level of $+G_z$ exposure. As the $+G_z$ level increased from +10 to $35G_z$ (exposure duration 30 s), a decrease in glucose, Cr-P, ATP, and CBV, and an increase in the level of lactate were observed (Fig.2). A decrease in CBF would decrease delievery of oxygen and glucose to the brain. This would lead to anaerobic glycolysis resulting in an increased lactate production. Although the in ATP and Cr-P levels correlates with the start of changes in the EEG (Fig. 1). A direct of decrease in ATP levels in causing I-EEG is not fully established. However, in non-centrifuge ischemic models it has been shown that EEG changes preceed any substantial decrease in ATP (1, 6). Also EEG recovery occurs before ATP levels are restored to control levels. In the present also although a significant decrease in Cr-P (47%) was evident at the time of I-EEG (15s), a reduction in ATP was not significant until 35 s (Fig. 3b). These observations question a direct role of ATP in causing I-EEG and it is likely that other factors may also be involved. On the other hand, due to compartmetation of ATP (27) it is speculated that its level may decrease below the threshold needed to support normal neuronal activity and this inturn may initiate a reduction of neuronal activity to conserve energy (10).

Increasing acceleration stress causes ischemia as evident by the reduction in CBV(Fig. 4). Determination of CBV was done by measuring the total iron content of the brain. Since there is a high degree of correlation between CBV and CBF changes (16) this simple method provides useful information about CBF. In addition, since the total iron and other metabolites can be measured in the same brain tissue a better correlation between the two can be investigated. The small but significant decreases in CBV as the $+G_z$ level increased $> +25G_z$ (26.3%) to $+35G_z$ (37%) were surprising since the presence of I-EEG at $+35G_z$ and metabolic alterations suggests global cerebral ischemia. The threshold for I-EEG is when CBF is reduced to below 25% of the control (9). In rats exposed to $+25G_z$ resulting in G-LOC, CBF was reduced by 95% (24) but CBV decreased by only 45 to 50% (7). The reason for this discrepancy is not clear. It is speculated that a sudden high $+G_z$ exposure may cause the cerebral vasculature to collapse and blood to be "trapped". This trapped blood could be reflected in a mismatch between severely reduced CBF but relatively small decreases in CBV during $+G_z$ exposure.

The time course studies in the whole brain show that a $+35G_z$ exposure induced significant changes in Cr-P and adenosine within 5 s, while the changes in glucose and lactate required 15 s (Fig. 3a). The increase in adenosine level was both dependent on the $+G_z$ (Fig. 2b) and exposure

duration (Fig. 3b). The sharp decrease in adenosine concentration at the time of the termination of centrifuge run could result from " wash out" due to the resumption of CBF and postischemic hyperemia (Fig. 4). Adenosine has been hypothesized to be a "retaliatory " metabolite and modulates the release of neurotransmitters and protects against neuronal damage. Adenosine is produced under conditions of ATP depletion, from AMP, to act against the external factors causing ATP depletion and to increase the supply of oxygen and substrates. Adenosine is also a potent vasodilator and its elevation during ischemia suggests that it may be physiological regulator of CBF.

Previously, it has been suggested that +Gz exposure may not reduce CBF uniformly, instead, may show a gradient due to the hydrostatic column effect. For example, CBF may be reduced relatively more in the part of the et al. (1994) found no regional difference in CBF in baboons exposed to +3Gz in the centrifuge but did not G – LOC. The present study shows some regional variability in CBV during a +35Gz (causes G-LOC) exposure (Fig. 4). The largest decrease in CBV was in the HIP (75%)>CTX and BS (50%)> MB and the CERE (22-to 25%) during 30 s centrifugation. All regions except the CTX and the HIP showed hyperemia (>control) and returned to control level by 60 s (except the HIP) post-centrifugation. The reason for lack of hyperemia in the CTX and the HIP is not clear. It is likely that since the CTX is most distal to the heart, may be the first region undergo ischemia, and perhaps the last to show hyperemia. Based on our pervious observations of a > 95% reduction in CBF at the time of G-LOC in rats (24), it is speculated that the regional differences in CBV observed in mice brain could be due to the variability in the amount of " trapped" blood.

The brain is heterogeneous in its cellular makeup and function, thus it is conceivable that there may be regional metabolic differences in its response to external stress, eg. +Gz exposure. This was not supported by present observations eg. All the brain regions showed a decrease in glucose, Cr-P and an increase in lactate during centrifugation (Fig.5). However, the changes in ATP showed some regional variability, since its level decreased significantly only in the CTX and the HIP and not in other regions. High lactate accumulation in the CTX (5.2 and 4.5 fold) and the HIP (4.5 and 2.6 fold) at 30 and 60 s post- centrifugation could results from lack of a hyperemic response in these regions and thus slow clearance (Fig. 4). There were no major regional differences in the level of decrease in Cr-P and ATP except in the MB.

In summary, the results of this study show that acceleration stress depending upon the level and duration, causes significant alterations in glucose and energy metabolism consistent with cerebral ischemia which may cause I-EEG or G-LOC. These data do not fully support the hypothesis that high +G exposure will induce a " gradient" in CBF among the regions of the brain which are more distal to the heart. However, it does not completely rule out some role of hydrostatic column effect in causing regional differences in CBV and metabolites. Similarity of metabolic changes in the whole brain and in the brain regions suggest that acceleration stress of short duration causes global cerebral ischemia.

Our rodent-centrifuge model is non-invasive and reproducible. Its unique MWF capability allows study of brain physiology, metabolism and histology at any desired time during or after ischemia. This model could be very useful in studying the effect of ischemia. This model could be very useful in studying the effect of ischemia, of varying severity, of short duration and multiple ischemia and reperfusion episodes.

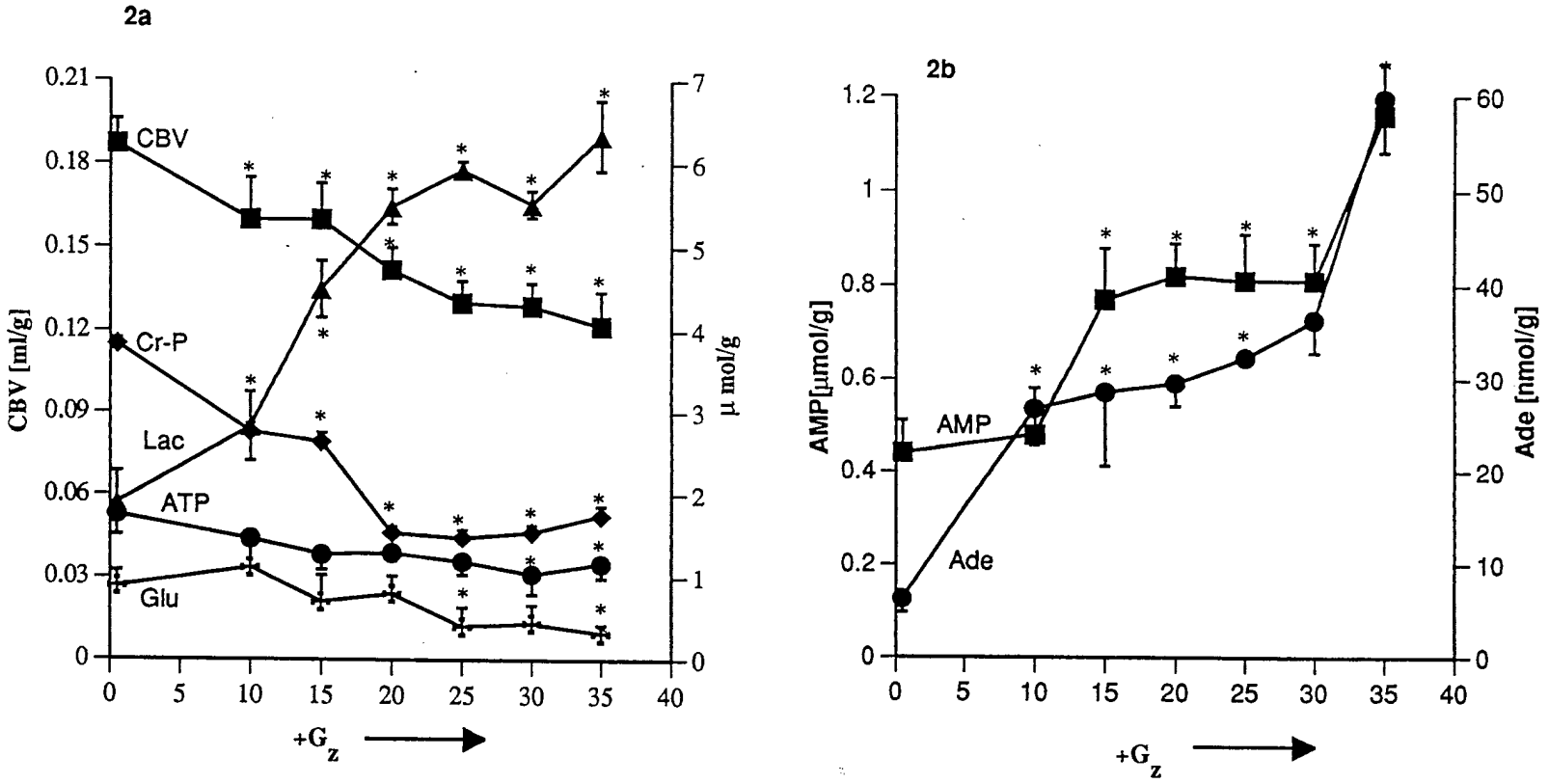

Fig. 2a, 2b: Effect of increasing $+G_z$ levels on CBV and metabolites in the whole brain. Mean ± SEM. * P <0.05, as compared to control (0.5 $+G_z$ exposure). Mice were exposed to Protocol 2 and metabolites were measured as described under methods.

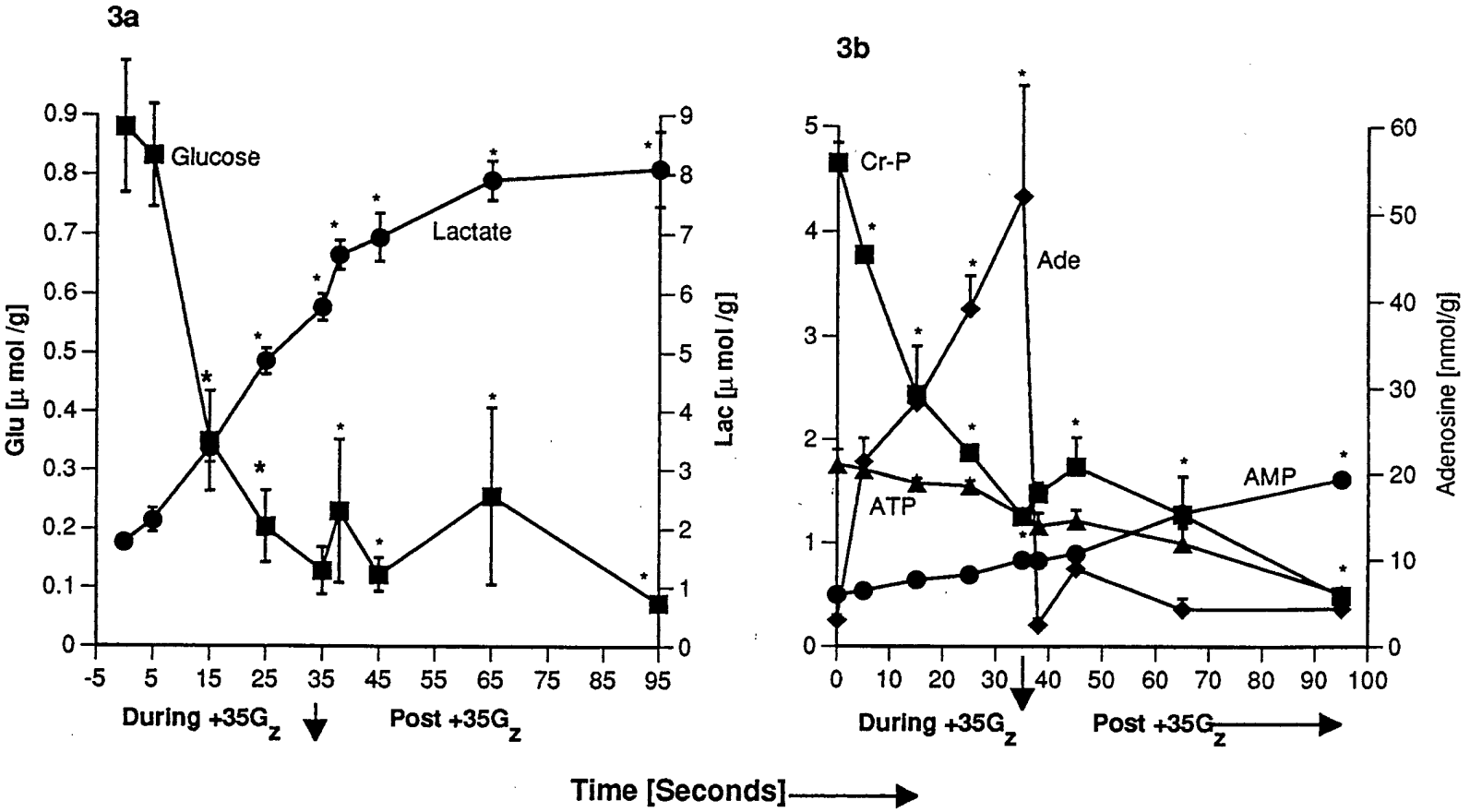

Figure 3 a, b: Time course of metabolites in whole mice brain. Mean ± SD, * P< 0.05 as compared to control. Mice were subjected to protocol 3, and whole brains samples were collected at indicated time points during and after +35G$_z$ exposure by microwave fixation.

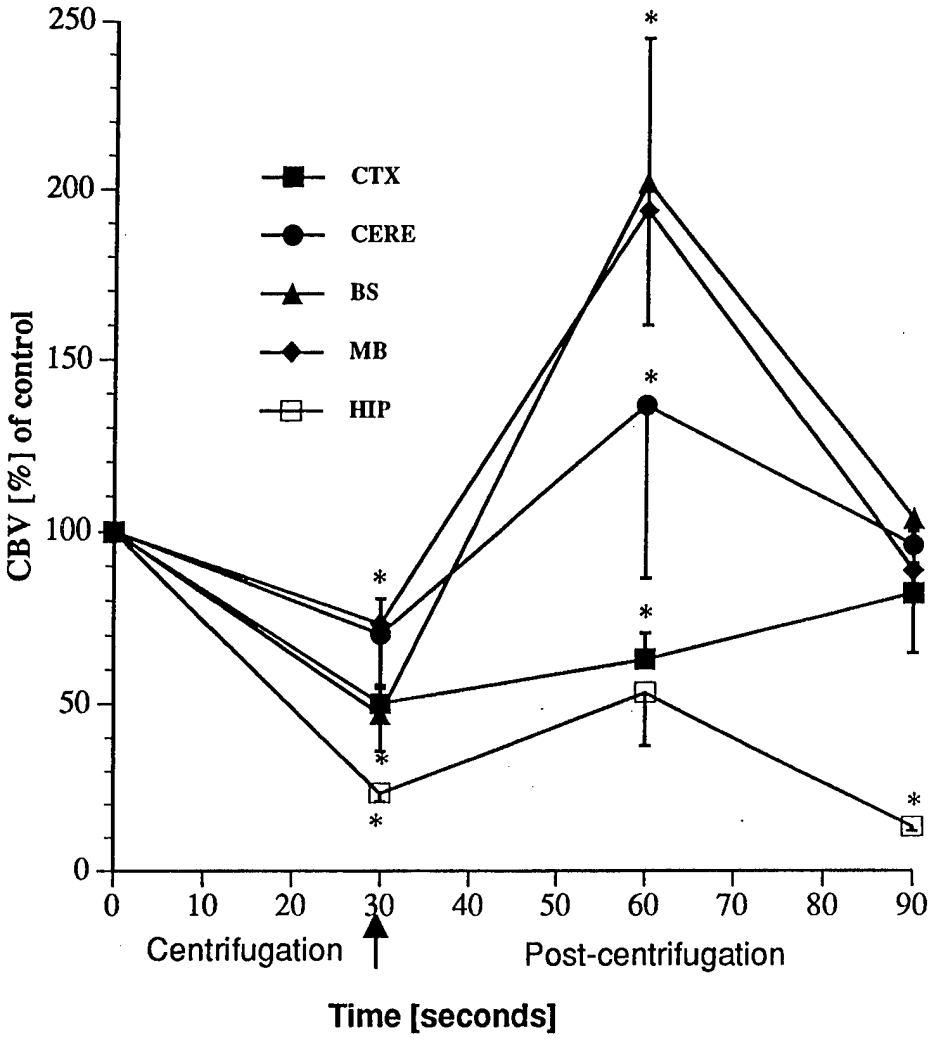

Figure 4: Time course of regional CBV. Data presented as % of control. Mean ± SEM (n=6-8), * P< 0.05 as compared to control. Mice were subjected to $+35G_z$ for 30 s and thebrain was fixed by MWF at the indicated. CBVwas determined by measuring the total iron content of the brain.

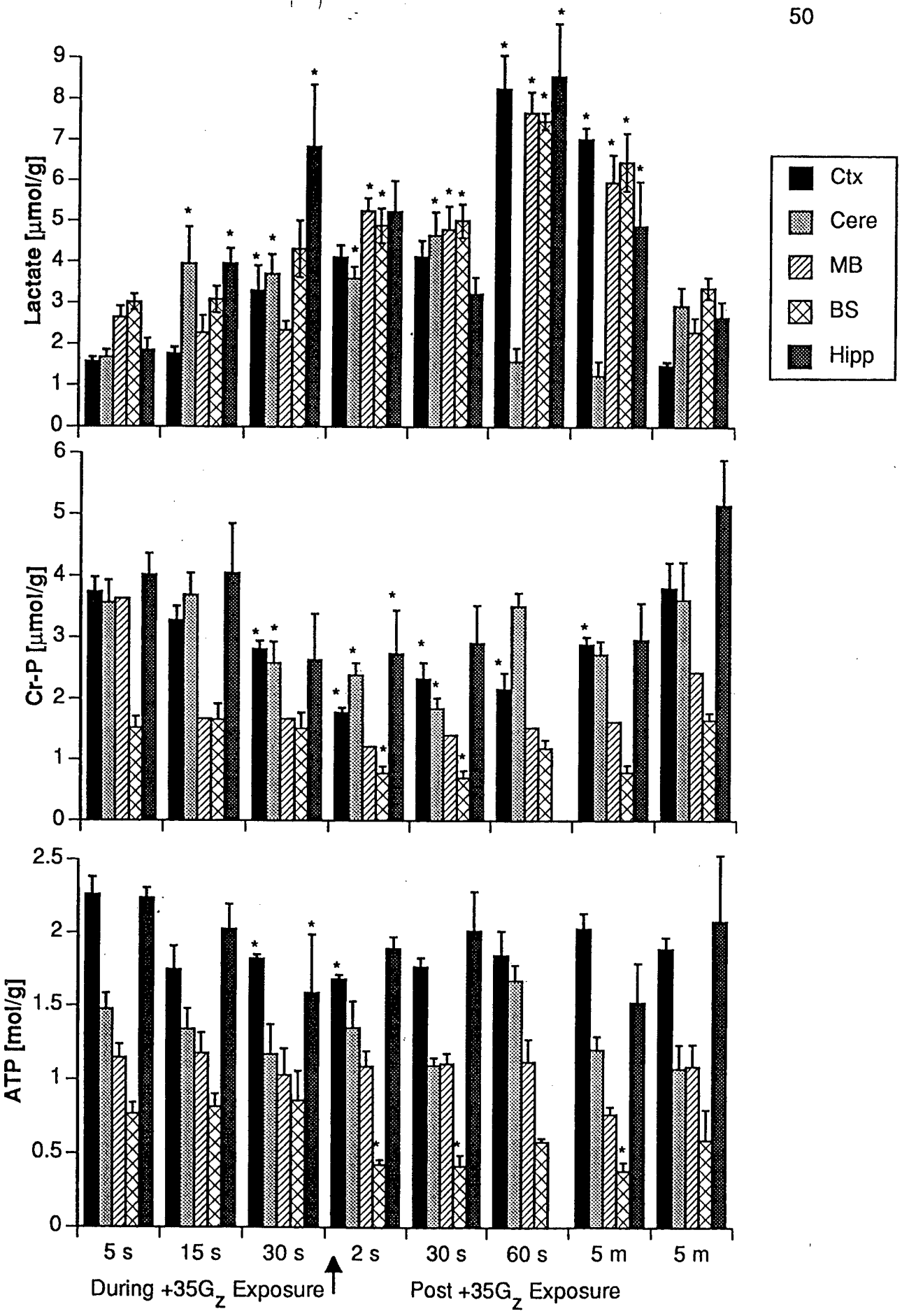

Figure 5 Mice brain regional metabolite changes during +35Gz exposure

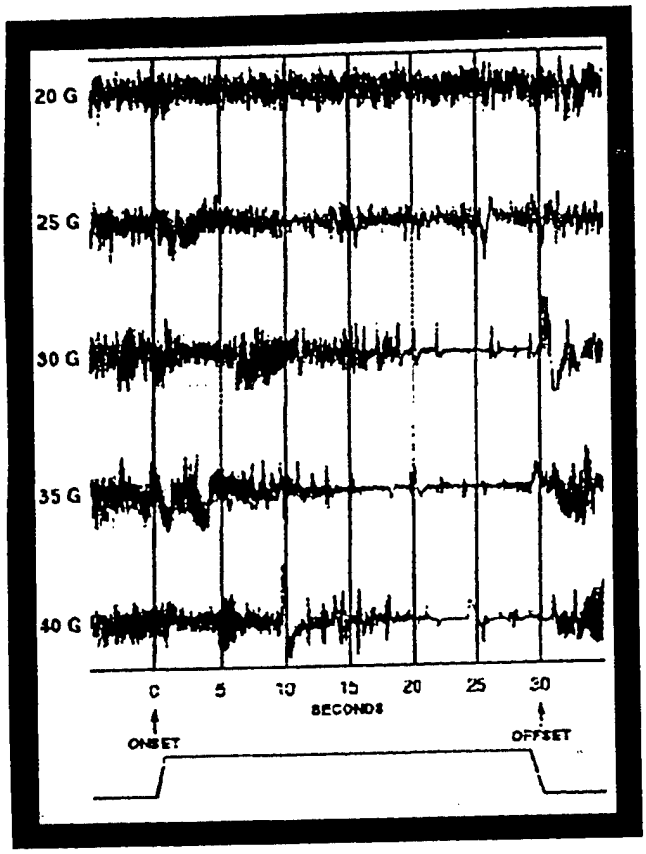

Figure 1: Effect of $+G_z$ exposure on mice EEG. Representative EEG tracings from one mice. Mice were implanted with biparietal EEG electrodes as described under Methods and exposed to indicated $+G_z$ levels for 30 s and EEG was monitored. Isoelectric EEG is defined as G-LOC.

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SECTION 7

This section describes the work done in the area of molecular neurobiology. The following two manuscripts have been submitted for publication.

Manuscript #1: submitted to the **Journal of Gravitational Physiology.**

Expression of c-fos, c-jun and HSP70 mRNA in Rat Brain Following Hypergravic Stress

Asha R. Shahed (Operational Technologies Corporation), Marjatta Son, John C. Lee (University of Texas Health Science Center) and Paul M. Werchan (Armstrong Laboratory, Brooks Air Force Base, TX)

Abstract:

Rats exposed to high $+G_z$ forces in a small animal centrifuge (SAC) exhibit loss of neuronal function (isoelectric EEG), termed G-induced loss of consciousness (G-LOC). This phenomenon is presumably due to a reduction in cerebral blood flow (CBF) or ischemia. Ischemia induces various metabolic and physiologic changes including expression of immediate early genes (IEGs) in the brain. Expression of IEGs have been suggested to be reliable markers for neuronal response to external stimuli or stress. In the present study expression of IEGs c-fos, c-jun and stress response gene HSP70 were measured in the brains of rats subjected to six 30 s exposures of $+22.5G_z$ in a small animal centrifuge. The level of c-fos, HSP70 and β-actin mRNA were measured by both Northern blot and RT-PCR. Expression of c-jun was measured only by RT-PCR. Expression of c-fos and c-jun was significantly stimulated at 0.5, 15, 30 and 60 min post-centrifugation. The level of HSP70 mRNA was significantly higher only at 60 and 180 min post-centrifugation. Measurement of metabolites showed a significant increase in lactate and a decrease in Cr-P level at 30 s and 15 min post-centrifugation, respectively. Lactate, but not Cr-P and ATP levels were restored to control levels by 60 min post-centrifugation. It is concluded that the transient expression of c-fos, c-jun and HSP70 mRNA is stimulated by repeated ischemic / reperfusion episodes induced by hypergravic stress.

Introduction;

The most severe effect of hypergravic stress in the head-to-foot vector ($+G_z$) is loss of consciousness (G-LOC). A high performance aircraft flying in a tight sustained circular pattern, exposes pilots to high acceleration forces in a head-to-foot direction. This centripetal acceleration results in an inertial reaction of equal but opposite force (G) that displaces organs and blood away from the brain and toward the extremities that can cause cerebral ischemia. Ischemia, under these conditions, is brief, repetitive, and can range from partial to complete depending on the level of $+G_z$ force. To understand the neurochemical correlates of $+G_z$ exposure and the mechanism of G-LOC, we have developed a rodent-small animal centrifuge (SAC) model. Utilizing this model we have shown that a 30 s exposure of $> +20G_z$ causes complete global ischemia signified by an

isoelectric EEG (I-EEG) or G-LOC [30, 34].

Cerebral ischemia, depending on the duration and severity, can induce a variety of cellular changes including edema, neuronal dysfunction, or neuronal death. Cerebral ischemia of 60 min duration induces reversible alterations in energy metabolism and electrophysiological function while ischemia of more than 2 min duration can cause brain injury [31]. In general $+G_z$-induced ischemia is of short duration (< 1 min) but can be repetitive. In experimental non-centrifuge models, multiple ischemic/reperfusion episodes show evidence of cumulative effects [24, 26]. Recent work from our laboratory has shown that six exposures of + 25 G_z (30 s each) or +10 G_z (2 min each) causes brain edema [29]. Also, daily 30 s exposures of +22.5 G_z for six weeks, resulted in neuronal death in the CA1 region of the rat hippocampus [33]. The +10 G_z and +25 G_z levels cause partial to complete global cerebral ischemia as indicated by metabolic changes and an I-EEG [29].

Recently, changes in gene expression have been used as reliable markers for neuronal response to external stimuli or stress eg. ischemia. Numerous studies have shown that cerebral ischemia induces the expression of immediate early genes (IEGs) c-fos and c-*jun* [2,7,9, 12,14,19, 25,36]. Similarly the expression of FOS, the primary response protein of c-fos gene was also increased by various stimuli e.g. cortical devascularization and ischemia [8], electrical stimulation [16] stress and seizures [27]. Long duration (60-90 min) hypergravic (+2G) stress has been shown to stimulate FOS protein expression in vestibular neurons. The IEG's, often referred to as third messengers, are suggested to play a role in the coupling of short term cellular stimuli to long term cellular response [4]. The IEG c-fos and its protein FOS may be an integral part of the signal transduction pathways [5,18]. Since c-fos expression can potentially alter the expression of other genes, it may be a part of the cell's survival mechanism in response to injury. Cerebral ischemia of greater than 2 min duration also stimulates the expression of stress gene HSP70 [1, 3, 11, 20-22]. HSP70, a major inducible stress gene, induction has been recognized as a marker for cell injury [23] and stress proteins may be essential for cellular recovery from injury, and ischemic tolerance [13].

In the present study, the expression of *c-fos, c-jun* and HSP 70 protein mRNA was measured in rat brains at various times following six, 30s exposures of +22.5G_z in the SAC by northern blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR).

Materials and Methods:

Animals: Male Sprague-Dawley adult rats (300-400 g) were provided free access to food and water and housed in an AAALAC approved facility.

+G_z exposure in the Small Animal Centrifuge (SAC): The SAC is 5' in diameter and 5' tall. It has a 21" head to axis radius and can reach +1-85G_z with an onset/offset acceleration rate of +10.4G_z/sec [34]. Seven rats can be centrifuged simultaneously, and eight channels of physiological data eg. EEG, ECG, CBF, and +G_z profile can be collected, analyzed and stored. Fully awake rats in a supine position were placed in individual plexiglas restraint cages and loaded on the SAC arm. The head was anchored on a bite bar with the nose facing the center shaft for a +G_z orientation. A foam cushion and a rigid metal plate was placed behind the buttocks to provide support during +G_z exposure. The SAC arm is fitted with a freeze fixation device allowing brain tissue removal in < 1 s at any time before, during or post-centrifugation for subsequent analysis [34].

$+G_z$ exposure protocol: Six groups (n=6/group) of rats were exposed to six, 30 sec exposures of $+22.5G_z$ each. A 5 min rest period was allowed between each run. Whole brains were removed following decapitation either at 0.5, 15, 30, 180 min or 24 hr post-centrifugation, placed in liquid N_2, pulverized and stored at -70 ^{0}C. The control rats (n=6) were exposed to six 30 s exposures of $+0.5G_z$ each, 5 min apart to incorporate stresses imposed by restraint and noise. The brains were collected 15 min post-centrifugation. An additional control group of rats (n=6) was restrained but not centrifuged. For the measurement of metabolites separate groups of rats were treated exactly as described above except that the brains were collected by freeze fixation at 30s, 15 and 60 min post-centrifugation.

Isolation of total RNA: Typically 0.1 g of frozen brain tissue was homogenized by Polytron (two 15 s bursts) in 2 ml of RNAzol™ B (Biotecx Laboratories, INC., Houston, TX) and 0.2 ml of chloroform was added and shaken vigorously for 15 s. After 15 min on ice the suspension was centrifuged for 15 min at 12,000 g (4^{0}C). RNA was precipitated by adding an equal volume of isopropanol to the aqueous phase. After 1 hr at 4^{0}C, RNA was collected by centrifugation. The pellet was washed twice with cold 75% ethanol, dried *in vacuo* , solubilized in 0.5 % SDS and stored at -70^{0}C. About 60 to 100 µg of high quality total RNA (260/280 ratio of 1.8-2.0) is obtained from 100 mg of tissue.

Northern blot analysis of c-fos mRNA in rat brain by ^{32}P labelled oligonucleotide probe: About 20 µg of total RNA was subjected to electrophoresis on 1% agarose gels (GTG grade, FMC, Bioproducts, Rockland, ME) containing 2.2 M formaldehyde (Sambrook et al., 1989). The gels were subsequently washed once in DEPC treated water for 15 min and twice in 10X SSC buffer for 20-30 min each at room temperature. The RNA was transferred on Nytran membranes (S&S maximum strength plus Nytran) overnight. The membranes were dried *in vacuo* for 2 hr and probed with a oligonucleotide (40 mer) specific for rat *c-fos* according to the manufacturer's protocol(Oncogene Sci. Inc.,Uniondale, NY). The 5'-labelled oligonucleotide was prepared in the presence of T4 polynucleotide kinase and [^{32}P] ATP, and purified on a Nensorb 20 (Dupont NEN Inc. Boston, MA) column. The blots were prehybridized (5ml / 50-100 cm^2) in sealed plastic bags in 1.0 M NaCL, 50 mM tris-HCL (pH 7.5), 10 % dextran sulphate, 1% SDS and 100 µg/ml single stranded salmon sperm DNA, for 5 hr at 65^{0}C. Blots were hybridized overnight in fresh pre-hybridization buffer containing ^{32}P-labelled probe (0.5 to 1.0 x 106 cpm/ ml). Post-hybridization washing consisted of four 15 min washes in 2 X SSC + 0.1 % SDS, one 45 min wash in the same buffer at 65^{0}C, a 5 min wash at room temperature, and a rinse in 2 X SSC. The blots were exposed on X-ray films and band intensity was quantified using a Phosphoimager (Molecular Dynamics, Sunnyvale, CA). The same blots were then stripped and reprobed with ^{32}P-labelled actin DNA probe (Oncogene Science Inc.). The c-fos/ ß-actin ratios were calculated from the band intensities of each band.

RT-PCR analysis of c-fos, c-jun and HSP70 mRNA in rat brain: One µg of total RNA was used for cDNA synthesis according to GENEAmp RNA PCR kit protocol except that the reaction was carried out at 42^{0}C for 60 min (Perkin Elmers Corp). The PCR reaction was performed exactly as described by the manufacturers, in the presence of cDNA, Taq DNA polymerase and 3' and 5' PCR primers specific for c-fos, c-jun or ß-actin (CLONTECH Laboratories Inc., Palo Alto, CA) or 3' and 5' PCR primers specific for HSP70 (Stressgene Inc., Vancouver, B.C.). The PCR products were subjected to 1.8% agarose gel electrophoresis and stained with ethidium bromide. The gels were photographed, scanned and the band intensity quantified by the NIH Image software. The length of PCR products were; 611 bp for c-fos, 411

bp for c-jun and a 236 bp for HSP70. To confirm the specificity of the PCR products, southern blots were probed with biotinylated plasmids containing c-fos or HSP70 sequences. The intensity of c-fos, HSP70, or c-jun PCR products were normalized to that of ß-actin and respective ratios were calculated.

Assay of Metabolites: The frozen brain tissue (0.2g) was homogenized in 2 ml of 7% cold perchloric acid using a Polytron homogenizer and centrifuged for 10 min at 10, 000 rpm. The supernatant was neutralized with KOH. Lactate and glucose were assayed as previously described (29). An aliquot (100 to 200 µl) of the supernatant was used to measure creatine phosphate (Cr-P) and adenine nucleotides by ion pair reversed phase high performance liquid chromatography (HPLC) on a Waters NOVA PAK C_{18} column exactly as previously described (34).

Statistics: The data were analyzed by one-way analysis of variance (ANOVA) and Dunnett's follow-up test for multiple comparisons. All data are presented as means ± SD of six animals.

Results:

Expression of c-fos and HSP70 mRNA (northern blots): The selection of $+22.5G_z$ exposures was based on previous observations that this level caused G-LOC or complete global ischemia in rats [30]. Rats were exposed to six $+G_z$ exposures because this treatment caused rat brain edema [29]. Northern blots of c-fos and ß-actin mRNA are shown in Fig. 1a. The c-fos /ß-actin ratios were calculated from the respective band densities to correct for any gel loading errors (Fig. 1b). Exposure to $+22.5G_z$ lacked significant effect on the expression of ß-actin at any time point. A major c-fos band and a faint band migrating slightly slower were detected. The intensity of the minor band correlated directly to that of the major c-fos band. However, the nature of this band is unclear at present and thus was not included in subsequent calculations. The data show that six $+0.5G_z$ exposures lacked significant effect on c-fos expression over the non-centrifuged control group. A significant increase in c-fos expression was observed 0.5 (2.7 fold), 15, 30 and 60 min following six exposures at $+22.5G_z$. The expression of c-fos was not significantly different at 180 min and was lower than the control 24 hr post-centrifugation. The 0.5 min post-centrifugation point represents a 30 min time lapse from the first $+22.5G_z$ exposure due to the 5 min rest period between each run.

The northern blots of HSP70 expression are shown in Fig. 2. HSP 70 mRNA bands show trailing and were not very sharp in either fresh blots or when stripped (hybridized for c-fos and stripped) blots were used. A significant increase in HSP70 mRNA expression was observed at 60 and 180 min post-centrifugation that returned to control levels by 24 hr.

RT-PCR analysis: Since northern blot analysis is a long process we used RT-PCR also to see if the results of both methods are comparable. The PCR products of c-fos, c-jun, HSP70 and β-actin mRNA are shown in Fig. 3a, 3b. Like the northern blots an increase in a 611 bp long c-fos product was observed at 30s, 15, 30 and 60 min post-centrifugation. The results of HSP70 mRNA (234bp) are similar to northern blots. Expression of c-jun was measured only by RT-PCR and a 410bp long product was detected. Like c-fos a significant increase in c-jun was observed at 30s, 15, 30 and 60 min post-centrifugation that returned to control levels by 180 min (Fig. 3b).

Effect of six, $+22.5G_z$ (30 s each) exposure on brain physiology and metabolism: The data in Table 1 show no significant differences in brain metabolites at 30 s, 15 and 60 min after six exposures at $+0.5G_z$ (control). No differences in glucose levels were detected at any time point following six $+22.5G_z$ exposures. In contrast, lactate level was elevated significantly at 30 s

(5 fold) and 15 min (3 fold) post-centrifugation that returned to control level at the 60 min time point. The level of creatine phosphate (Cr-P) was lower than controls at each time point. ATP levels were not significantly different than control at 30 s and 15 min time points but were lower at the 60 min time point.

Discussion:

Hypergravic stress, depending upon the severity and duration, can impose both a metabolic and perhaps a physical stress due to movement of the brain. A single exposure of >+20 G_z caused > 95% reduction in CBF [35], accumulation of lactate, decrease in energy metabolites and isoelectric EEG [30]. It was concluded that a single >+20 G_z (30 s) exposure causes global cerebral ischemia. Similar metabolic changes were observed following six exposures of +22.5G_z suggesting rats were subjected to six ischemic /reperfusion episodes (Table 1).

The result of this study demonstrated that six (30 s each) brief exposures of +22.5G_z in the SAC increased the expression of c-fos, c-jun and HSP70 genes. The increase in gene expression was immediate and transient. The largest increase in c-fos and c-jun expression was observed immediately after the last run and maintained for up to 60 min post-centrifugation. In contrast, a significant increase in HSP 70 expression required longer reperfusion duration (60 and 180 min post-centrifugation. This time course was similar to the reported studies using non-centrifuge models of global ischemia [2, 9, 12]. The ischemic duration threshold for c-fos and HSP70 expression has been reported to be between 1 and 2 min in gerbils [9, 12]. This ischemic duration corresponds to energy depletion and membrane depolarization in the brain. In the present study 30 s ischemic events (+G_z exposures) were repeated six times with 5 min of reperfusion. Data in Table 1 illustrate that complete metabolic recovery did not occur until 60 min post-centrifugation. To our knowledge there have been no previous reports where the effect of less than 1 min of ischemia on c-fos or HSP70 expression has been investigated.

Ischemia-induced gene expression was observed under conditions that also caused neuronal damage [1, 2, 9, 14]. However, neuronal damage reportedly occurs when ischemic duration is > 2 min [15]. As reported above, c-fos and HSP70 expression can be stimulated by 1 or 2 min of ischemia. Previously we have reported that six high +G_z exposures caused brain edema, 15 to 60 min post-centrifugation, that resolved within 3 hrs [29]. Interestingly, the time points of gene expression are almost identical to edema formation. However, it remains to be determined whether this treatment causes any neuronal damage. In non-centrifuge models of global ischemia multiple ischemic episodes resulted in worsend neuronal outcome than a single episode of similar duration [24-26]. In these studies ischemic duration was much longer (3 min) than the 30 s in the present study. However, it is possible that high +G_z exposure, in addition to the ischemic stress, could impose a mechnical stress due to the high onset rates (10.4 G/s), that could cause displacement of brain and other organs. Potential contributions, if any, and the extent of this insult to the observed increase in the level of c-fos expression remain to be investigated. Two reports have shown that 60-90 min exposure of rats to +2G increased FOS expression [7-10]. This G level is not likely to cause ischemia suggesting exposure duration to be a stress factor. Since the exposure duration in the present study was only 30 s (180 s total for six exposures) it is suggested that +G_z-induced ischemia may be the primary factor in causing enhanced gene expression.

Cerebral ischemia of short duration stimulates strong expression of stress proteins [1,5, 22]. *In situ* hybridization studies revealed that the expression of HSPs was stimulated in cell populations where CBF was reduced to below 50% during focal ischemia. These cells, although

metabolically compromised, do not die. The role of HSP 70 expression following ischemia is not fully understood. Some recent evidence suggests that they may be involved in ischemic tolerance and may provide protection from ischemic damage [11,13]. As stated above, the expression of IEG's and HSP70 expression is investigated under ischemic conditions designed to cause neuronal damage. Since we speculate that conditions used in the present study are not sufficient to cause neuronal damage, the expression of IEGs and HSP70 genes is difficult to explain. It is speculated that the expression of these genes may be part of initial cellular response to external stress. The products of c-fos and c-jun oncogenes are known to form a complex that binds to the AP-1 site of DNA. Such a complex may be involved in the regulation of long term responses to external stimuli [28] by acting as a "master switch" to upregulate other genes involved in long term cellular responses [17]. However, for a definitive answer, histological evaluation of brain tissue following $+G_z$ exposure is needed.

A significant level of c-fos mRNA was found in the brains of all control animals suggesting a physiological role for these genes [18]. The level of c-fos, c-jun and HSP70 gene expression was unaffected by centrifugation at $+0.5G_z$ (Figure 1a). Exposure to $+0.5G_z$ level lacked a detectable effect on either brain metabolism or CBF, compared to non-centrifuged rats [33]. Therefore, we routinely exposed control rats to $+0.5G_z$ to incorporate possible stresses imposed by restraint and noise. Expression of c-fos and HSP70 mRNA were examined by both northern blot and RT-PCR analysis with very similar results (Figs 1, 3). The specificity of PCR products were confirmed by southern blot analysis with either biotinylated plasmid probes or ^{32}P-labelled oligonucleotide probes (data not shown). These observations suggest that the RT-PCR method is a valuable alternative to more time consuming northern blot analysis of gene expression

In summary, these results, for the first time, show that six 30 s exposures of $+22.5\ G_z$ each, stimulate expression of c-fos, c-jun and HSP 70 genes in the whole rat brain. It is speculated that the increased gene expression is the result of cumulative insult of multiple $+G_z$ exposure-induced ischemia / reperfusion episodes.

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2. The animals involved in this study were procured, maintained and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the institute of Laboratory Animal Resources - National Research Council. Armstrong Laboratory is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).
3. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Air Force.
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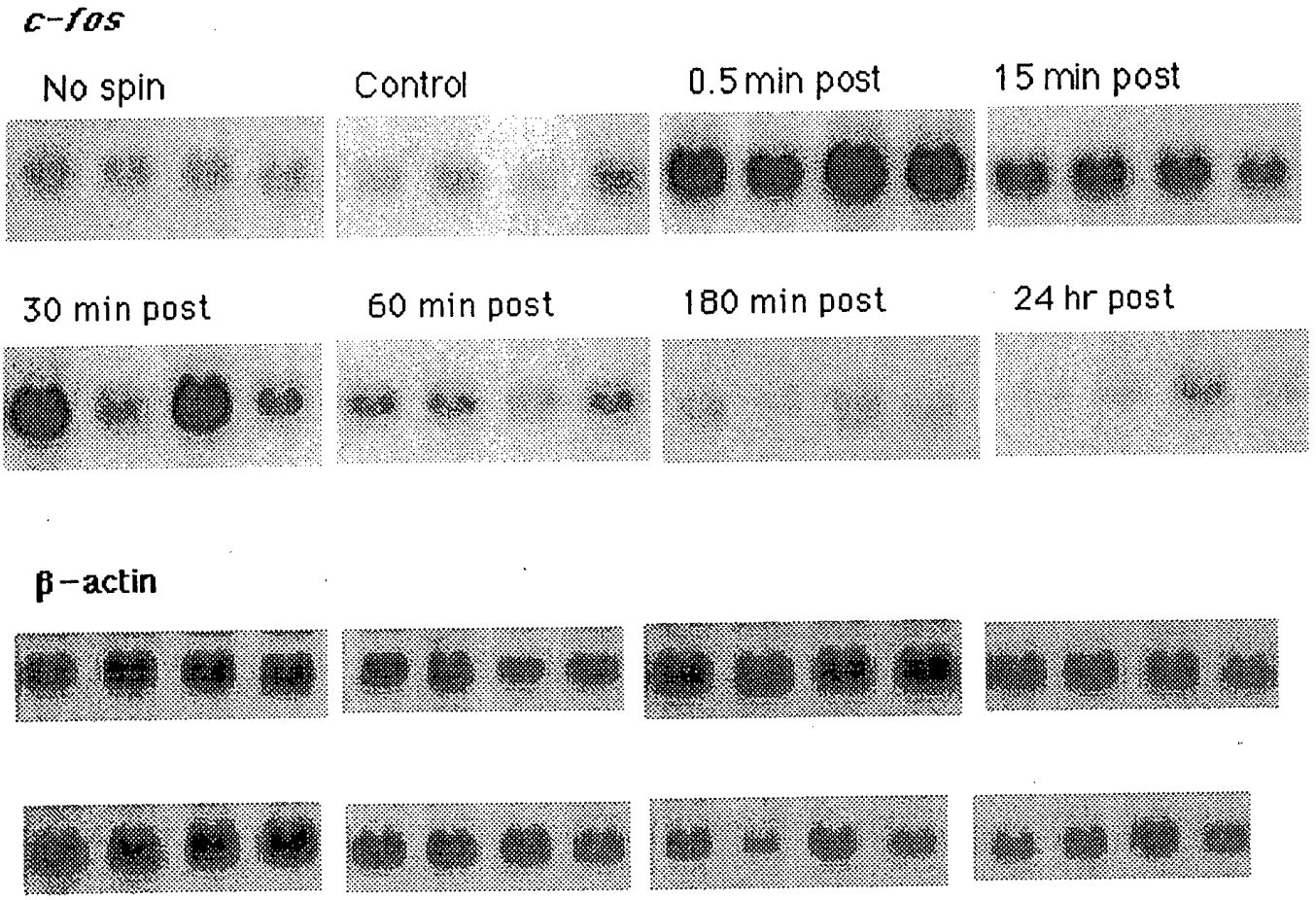

Fig. 1b

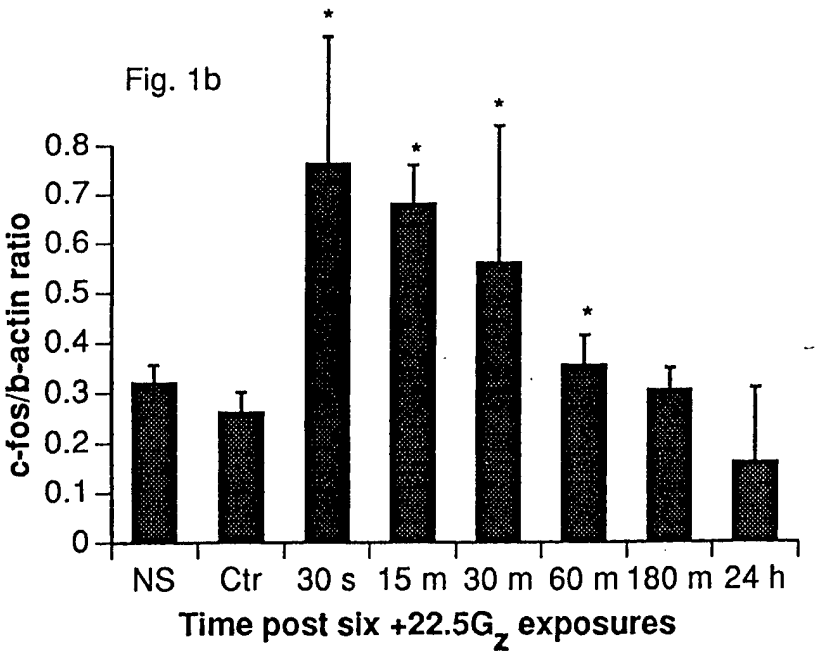

Figure 1a and 1b: Expression of c-fos and beta actin in rat brain following six 30 s exposures at +22.5Gz. Figure 1b shows the the ratios of c-fos/actin.

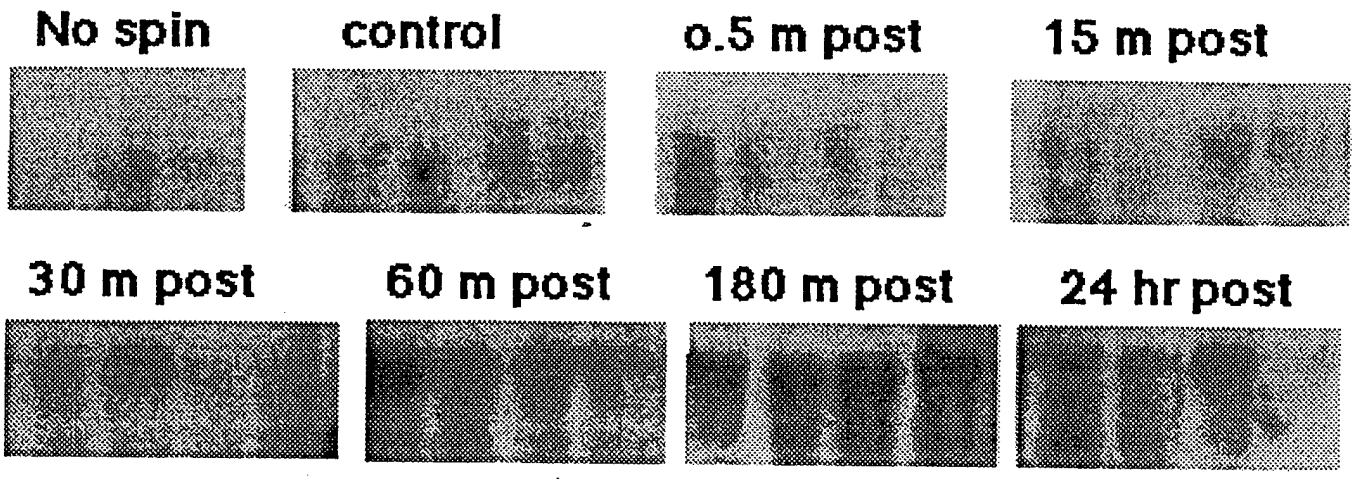

Figure 2: Northern blots of HSP70 mRNA in rat brain: Experimental details are exactly same as in Figure 1. A ^{32}P labelled oligonucleotide specific for HSP 70 was used for hybridization.

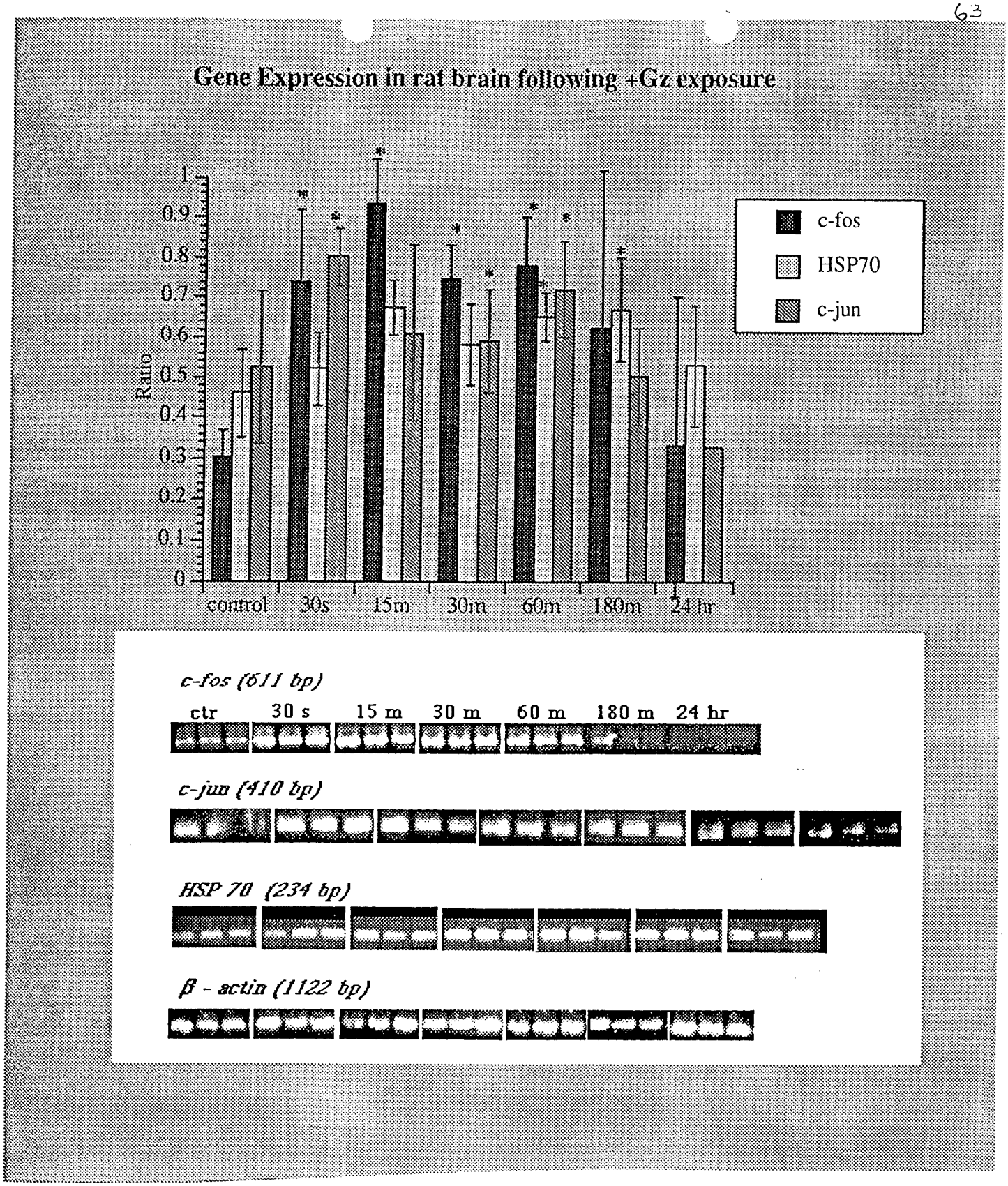

Figure 3a and 3b: Expression of c-fos, c-jun, HSP70 and β-actin mRNA in rat brain by RT-PCR analysis. Total RNA used for northern blots was used for cDNA synthesis and specific 3' and 5' primers were used for PCR reaction exactly as described under methods. Only the representative gels of PCR lanes are shown. Figure 2b shows the ratios of c-fos, c-jun and HSP70 mRNA's with β-actin.

Table 1

Effect of Six Exposures of +22.5G_z on Rat Brain Metabolites

Protocol	Glucose	Lactate	Cr-P	ATP
6 X +0.5G_z				
30 s post	2.08 ± 0.5	0.96 ± 0.2	3.14 ± 0.4	1.68 ± 0.2
15 m post	2.76 ± 0.4	0.75 ± 0.1	3.01 ± 0.6	1.85 ± 0.2
60 m post	2.40 ± 0.3	0.76 ± 0.2	2.94 ± 0.2	1.67 ± 0.04
6 X +22.5G_z				
30 s post	2.81 ± 0.2	5.03 ± 0.06*	2.41 ± 0.1*	1.52 ± 0.06
15 m post	2.84 ± 0.4	3.06 ± 0.7 *	1.78 ± 0.9	1.62 ± 0.7
60 m post	2.23 ± 0.02	0.71 ± 0.1	0.57 ± 0.05	1.07 ± 0.4

Mean (μ mol/g wet weight) ± SEM (n=3/group), *P < 0.05 as compared to control. Control groups were exposed to six exposures of 0.5G_z (30 s each) with a 5 min rest period between runs. Brains were collected by freeze fixation at indicated time points after the last centrifuge run. Experimental groups were treated identically at +22.5G_z.

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Manuscript # 2 submitted to **Neuroscience Letters.**

Brief High $+G_z$ Exposures in a Small Animal Centrifuge Stimulates*c-fos* and HSP70 Gene Expression in Rat Brain

Marjatta Son, **Asha R. Shahed**[1], Paul M. Werchan[3] and John. C. Lee
Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX, U.S.A. 78284-7760, Operational Technologies Corp[1], Armstrong Laboratory[3], Crew Technology Division, Brooks Air Force Base, TX 78235

Summary:

High $+G_z$ exposure (head-to-foot inertial load) has recently been shown to cause global cerebral ischemia. In the present study, expression of an immediate early gene, *c-fos* and heat shock protein, HSP70 gene in response to $+22.5G_z$ exposure was investigated in rat brain by Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Adult rats were subjected to a single exposure of $+22.5G_z$ for either 60s (Protocol 1), 15 or 30 s (Protocol 2) in a custom built small animal centrifuge (SAC). Brains were removed at various time points (0.5 min to 24 hr) after $+22.5G_z$ exposure by either decapitation or freeze-fixation. Control rats were exposed to $+0.5G_z$ for the corresponding duration. Exposures to $+22.5G_z$ for either 15 or 30 s were sufficient to cause a small but significant increase in *c-fos* mRNA level 30 min post-exposure. The level of HSP70 mRNA remained unchanged. After exposure to $+22.5G_z$ for 60 s, the level of *c-fos* mRNA was significantly elevated by 15 min post-exposure, remained elevated until 60 min, and returned to the control level later. An increase in HSP70 mRNA level was not observed until 60min post-exposure and remianed elevated until 180 min. A significant decrease in the level of creatine phosphate (Cr-P) and ATP and an increase in lactate was detected during 60 s exposure to $+22.5G_z$. These metabolites returned to the control levels post-exposure. On the basis of these observations, it is proposed that the 60s $+22.5G_z$ exposure caused global cerebral ischemia, which, in turn, induced *c-fos* and HSP70 expression. The early and transient increase in *c-fos* and subsequent increase in HSP70 epxression should serve as useful biomarkers for investigations of potential effects of hypergravic stress on the brain.

Introduction:

High $+G_z$ exposure has been known to cause a transient loss of consciousness (G-LOC) in pilots of high performance aircraft. G-LOC has been proposed to result from a sudden reduction in cerebral blood flow (Burton, 1988). To understand the neurochemical effects of high $+G_z$ exposures and mechanism of G-LOC, a rodent-small animal centrifuge (SAC) model was developed (Werchan and Shahed, 1992). In this model, a 30 s exposure of $+25G_z$ was shown to cause transient global cerebral ischemia, significant alterations in the levels of energy metabolites, and an isoelectric EEG. EEG recovery occurs within 15 to 45 s following $+G_z$ exposure, whereas complete metabolic recovery requires 3 to 15 min.

Relatively little is known about post $+G_z$ exposure effects on brain metabolism and function. Recently we have shown that multiple $+G_z$ exposures cause brain edema, one of the earliest manifestations of transient cerebral ischemia (Shahed et al., 1994). Depending upon the severity and duration, ischemia can cause a variety of additional physiological, morphological and biochemical effects (Milde, 1989; Siesjo, 1992).

In recent years, cerebral ischemia has been shown to alter gene expression during reperfusion in rodent brains (for a review see Chopp, 1993; Kogure and Kato, 1993). The expression of immediate early genes (IEG"s), c-fos and c-jun has been shown to occur in rodents following 1 to 90 min of cerebral ischemia (An et al., 1993; Kindy et al. 1991; Nowak et al., 1990; Onodera et al. 1989; Welsh et al., 1992; Wessel et al., 1991). The change in gene expression appears to be the result of an increased trnascriptional rate of the *c-fos* gene, and not a change in the *c-fos* mRNA stability (An et al., 1993). Two studies have shown increased synthesis of FOS protein in neurons of the vestibular system as well as in several other areas of the rat brain following 60 to 90 min exposure at $+2G_z$ in a centrifuge (Fuller et al., 1994; Kaufmann et al., 1992). FOS has been shown to activate or repress a number of late effector genes that are thought to modulate neuronal response to injury (Hengerer et al., 1990; Sonnenberg et al., 1989). Alterations in gene expression may occur to prevent post-ischemic alterations that could lead to cell death (Araki et al., 1990). Besides ischemia, a variety of other stimuli have been reported to induce expression ofIEG,s (Wessel et al., 1991 and references therein).

Cerebral ischemia has also been shown to stimulate the expression of stress genes. Stress proteins appear to be essential for the recovery of cells after injury. HSP70 is a major inducible stress gene, and its induction has been recognized as a marker for cell injury (Gonzalez et al.,1989). The expression of HSP70 gene has been detected after cerebral ischemia of at least 2 min duration (Abe et al., 1991; Aoki et al.,1993; Kawagoe et al., 1992; Nowak et al., 1990; Welch et al., 1992). Although the role of HSP70 in cerebral ischemia is not completely known, recent evidence suggests that HSP70 might be involved in ischemic tolerance (Kirino et al.,1991; Kitawaga et al.,1991).

We hypothesized that $+G_z$ exposures would affect gene expression, since $+G_z$ exposure causes global ischemia and may also exert physical insult, such as movement of the brain. In the present study, the expression of*c-fos* and HSP70 mRNA in the brains of rats following varying duration of $+22.5G_z$ exposures in the SAC were measured by Northern blot and by RT-PCR analysis.

Materials and methods:

Animals: Male adult 300-400g Sprague-Dawley CD-VAF/Plus rats (Charles River Laboratories, Wilmington, MA), were provided free access to food and water both before and after centrifugation.

+G_z exposure protocols:

Protocol 1: Six groups of rats (n=5-6/group) were subjected to a single 60 s exposure at +22.5G_z. The rats were decapitated at immediately after,15, 30, 60, 180 min, or 24 h post-centrifugation. Brains were removed, pulverized in liquid N_2 and stored at -70°C. Another set of rats were treated identically except that the brains were collected by freeze-fixation to permit measurements of metabolites.

Two groups of controls were used. One group of rats (n=6) was centrifuged at +0.5 G_z for 60 s to subject the rats to stresses imposed by restrain, noise and vibrations of the SAC. The second group was restrained but not subjected to centrifugation. Brains from both groups were collected after 15 min of manipulation.

Protocol 2: Two group of rats (n=5/group) were exposed to +22.5G_z for 15 s, and the brains were collected by freeze-fixation at 30 or 60 min after centrifugation. Three additional groups of rats (n=5/group) were exposed to +22.5G_z for 30 s, and the brains were collected at 30, 60 or180 min post-centrifugation. Three groups of control rats (n=5/group) were centrifuged at +0.5 G_z for 30 s, and brains were collected at 30, 60 or 180 min. Brain tissues were pulverized and stored at -70°C.

Isolation of total RNA and the details of northern blot analysis were exactly the same as in manuscript 1 in section 7 and therefore are deleted from this one for the purpose of this report.

Results:

Effects of a 60 s +22.5 G_z exposure on the expression of *c-fos* mRNA or HSP70 mRNA (Protocol 1): Typical Northern blots of*c-fos* and ß-actin mRNA are shown in Fig. 1A and 1B, respectively. A significant stimulation in *c-fos* gene expression was observed in the rat brain following a single 60 s exposure at +22.5G_z. A faint band migrating slower that the main *c-fos* band was also detected. The intensity of the minor band correlated directly to that of the major *c-fos* band. However, the nature of this band is unclear at present and thus was not included in subsequent calculations. No significant changes in the expression of b-actin gene was observed (Fig. 1B). To facilitate comparison of data values for *c-fos* were normalized to those of ß-actin (Fig. 1C).

The level of *c-fos* mRNA was not significantly different between the two groups of control rats, one of which was centrifuged for 60 s at +0.5G_z while the other was not (Fig. 1C). Based on this observation in the subsequent experiments control group of rats were routinely exposed to +0.5G_z. The level of *c-fos* expression was not statistically different from the control when brains were collected immediately after centrifugation. However, a significant increase was observed at 15, 30 and 60 min time points. The level of *c-fos* mRNA returned to the control level at 180 min and slightly below at the 24 h time point (Fig. 1A and C).

RNA samples from the same rats were also subjected to RT-PCR analysis for the determination of *c-fos* mRNA level. The results of RT-PCR analysis (Fig. 2) are remarkably similar to those obtained with the Northern blot analysis. The specificity of c-fos PCR product (611 bp) was confirmed by Southern blot analysis using a biotinylated c-fos DNA probe(3.2 kb). That these data agree well with each other indicates that the faster and simpler RT-PCR method can be an alternative to Northern blot analysis for studying gene expression.

Expression of HSP 70 mRNA was measured by RT-PCR (Fig. 3). The level of HSP70 mRNA was not significantly increased above control levels until at 60 min post-centrifugation. The level remained elevated at 180 min and declined thereafter. Similar results were obtained by Northern blot analysis (data not shown).

Effect of +22.5G_z exposure on rat brain metabolites: Rats were exposed according to protocol 1 and the brain was collected by freeze-fixation. A four fold increase in lactate concentration was observed at the 60 s time point during centrifugation (Fig.4A). The level of lactate returned to the control level at 15 min and with one exception, remained at that level until 24 h post- centrifugation. The exception occured at 30 min post-centrifugation where a secondary, two-fold increase in the lactate level was observed. The concentration of both Cr-P and ATP decreased significantly at the 60 s time point (Fig. 4A). Although the level of Cr-P was restored to the control level within 15 min, it took longer for the ATP level to return to the pre-centrifugation level. The concentration of adenosine was not changed (data not shown).

To estimate CBF during and post-centrifugation, total Fe content of the brain was determined and the data are shown in Fig. 4B. A greater than 50% decrease in total Fe at the 60 s time point (during centrifugation) and a secondary decrease at 30 min (post-centrifugation) were observed. At other time points, the Fe content was not significantly different from the control.

Effect of 15 s and 30 s +22.5G_z exposure on expression of *c-fos* mRNA(Protocol 2): These experiments were conducted to determine whether +G_z exposure of a shorter duration would effect *c-fos* expression. In Protocol 2 brain samples were collected by freeze fixation to allow measurement of metabolites in the same samples. The *c-fos*/ß-actin ratios are shown in Fig. 5. A detectible level of*c-fos* mRNA was found in all control groups subjected to either 15 or 30 s of centrifugation at +0.5G_z. However, the level of *c-fos* mRNA was significantly lower in brain samples collected at 60 and 180 min post-centrifugation compared to 30 min time point. The level of *c-fos* m RNA increased significantly 30 min following a 15 or 30 s exposure at +22.5G_z over the corresponding controls. But the level of *c-fos* no longer differed significantly after 60 or 180 min over the corresponding controls. The level of HSP70 expression was also measured in the same RNA samples and no significant increases over control values were detected (data not shown).

The level of metabolites and total Fe were also measured in the above brain samples. The level of lactate and Cr-P and ATP at 30, 60 and 180 min after either a 15 or 30 s exposure at +22.5 G_z were essentially the same as their corresponding controls (data not shown). Contrary to the rats exposed to +22.5G_z for 60 s (Fig. 3b), no evidence of secondary ischemia (as indicated by a decrease in total Fe content) was observed at 30 min after +22.5G_z exposure of either 15 or 30 s (data not shown).

Discussion:

Exposure to high +G_z, depending upon the level and duration, can potentially result in various physiological and perhaps morphological alterations. One of the acute effects of +G_z exposure is cerebral ischemia due to a pooling of blood away from the brain and towards the body extremities. We have previously shown that when rats are exposed to higher than +20G_z in the SAC, CBF decreases by 95% in less than 5 s (Werchan et al., 1993). Loss of neuronal functions or G-LOC (isoelectric EEG) occurs within 15 s (Werchan and Shahed, 1992). Cessation of CBF is followed by a characteristic hyperemic response within seconds after the termination of the SAC run. Results of the present study clearly show that a single 60 s, +22.5G_z exposure caused a

significant increase in *c-fos* and HSP70 expression in the rat brain. The observed alterations in the level of brain energy metabolites measured during a 60 s exposure of +22.5 G_z (see below) are consistent with symptoms of global cerebral ischemia. In light of these data, we propose that the increases in *c-fos* and HSP70 expression may be caused by +G_z-induced global cerebral ischemia.

Our data also showed a significant level of c-fos mRNA in the brains of all control rats which were neither anesthetized nor subjected to any surgical procedures. These observations agree with earlier suggestions that c-fos plays a physiological role in the normal brain (Morgan et al., 1987). Furthermore, a comparison of the level of *c-fos* mRNA in the two control groups which were identically treated except for centrifugation at +0.5G_z, suggests that this level of exposure has negligible effect on *c-fos* expression (Fig.1A and1C). Together with our previous observations that centrifugation at +0.5G_z does not affect the EEG, CBF or the level of metabolites (Werchan and Shahed, 1992), we feel that exposure to +0.5G_z is useful as a control for these studies, to incorporate any possible stresses imposed by restraint and noise.

The present study also revealed that the increase in *c-fos* mRNA level post-centrifugation at +22.5G_z occurred rapidly and transiently. The data agree with several other studies in which vessel occlusion models of cerebral ischemia were used (Kindy et al., 1991; Nowak et al., 1990; Onodera et al., 1989; Welsh et al., 1992; Wessel et al., 1991). Our data showed that *c-fos* mRNA levels reached a maximum at15-30 minpost +22.5Gz exposure. The level of *c-fos* mRNA remained significantly elevated at 60 min but declined to control levels by 180 min. The reason for this decline might be that *c-fos* mRNA is fairly unstable and is degraded rapidly (Morgan and Curran, 1991; Muller, et al., 1984). In addition, the *c-fos* gene may be repressed by the newly synthesized FOS protein and possibly by products of other immediate early genes (Morgan and Curran, 1991; Sassone-Corsi et al., 1988).

Our data on the level of lactate, Cr-P, ATP and total iron are consistent with the symptoms of global cerebral ischemia. Although the data show significant decrease in Cr-P, ATP, total iron content and an increase in lactate during the 60 s exposure of +22.5G_z, their levels restored to control levels by 15 min. Our observations of a secondary decrease in total iron content and an increase in lactate production at 30 min suggest the occurrence of secondary ischemia. The incidence of secondary ischemia has been reported in vessel occlusion models of ischemia (Kagstrom et al., 1983; Karlsson et al., 1994). The significance of secondary ischemia and its effect on gene expression is not clear. Furthermore, these observations provide an explanation for the lack of an increase in *c-fos* mRNA immediately after +22.5G_z exposure. It is likely that since the cellular energy levels were decreased at this point (immediately after) *c-fos* mRNA level did not increase. But at15 min post-centrifugation, metabolic recovery had occurred, and an increase in*c-fos* mRNA level was evident. This finding suggests that the resumption of CBF and subsequent restoration of cellular energy metabolites may be required before de novo *c-fos* mRNA synthesis can occur. Other studies have reported an increase in *c-fos* mRNA15 to 90 min after the initial ischemic insult (Kindy et al., 1991; Onodera et al., 1989; Wessel et al., 1991).

In all previous studies, the duration of ischemia was long, it varied from 1 to 90 min, with an average of 20 min. The level of*c-fos* expression was proportional to the duration of ischemia: the level of *c-fos* mRNA increased as the duration of ischemia was increased from 1 to 10 min in gerbils (Kindy et al.,1991). In the present study, the duration of +G_z induced ischemia was much shorter (15 to 60 s), but it was sufficient to induce*c-fos* expression. Itshould be pointed out that the present study was conducted in a centrifuge with very high +G_z onset rates. Therefore, it is likely that this environment may impose a physical force, e.g. movement of the brain. Potential contributions, if any, and the extent, of this insult to the observed increase in the level of *c-fos*

expression cannot be ascertained.

The expression of HSP70 occured later (60 min) and it lasted longer (180 min) than that of *c-fos* , in agreement with other studies(Abe et al. 1991; Aoki et al.1993; Kawagoe et al.,1992; Nowak 1991; Welsh et al., 1992). Several reports show that ischemia induced elevation of HSP70 mRNA is sustained longer in cell populations which do not recover from injury (Kawagoe et al.,1992; Nowak et al.,1990; Welsh et al.,1992). We did not detect such prolonged expression of HSP70 mRNA in the whole brain samples, since HSP70 mRNA expression returned to control levels within 24 hrs. In contrast to *c-fos*, expression of HSP70 was detected only after 60 s exposure, but not after 15 or 30 s exposures to $+22.5G_z$(data not shown). This is in agreement with findings that the induction of HSP70 may require a more serious insult than that needed for *c-fos* (Nowak et al.,1990).

The importance of increased *c-fos* or HSP70 expression following $+G_z$exposure or ischemia is not well understood. It has been suggested that the protein products of immediate early genes, *c-fos* and *c-jun* oncogenes form a complex that presumably acts as a "master switch" to upregulate other genes (Marx, 1987). The complex binds to the AP-1 site of target genes, some of which might be involved in the regulation of long term responses to external stimuli (Morgan and Curran, 1991; Rauscher et al. 1988). HSP70 gene does not have AP-1 binding site, and it is unlikely that activation of *c-fos* has a direct effect on HSP70 expression. However, the expression of HSP70 might be among the later waves of transcriptional activation triggered after the initial induction of *c-fos* (Nowak,1991; Welsh et al.,1992). A role of HSP70 expression in ischemic tolerance has also been suggested (Liu et al., 1992, Kirino et al., 1991).

In summary, the results of the present study show that a 15 to 60 s exposure to $+22.5G_z$ was sufficient to induce global cerebral ischemia (during centrifugation) and increase expression of *c-fos* mRNA 15 to 60 min post-centrifugation. The elevation in *c-fos* mRNA level was rapid and transient. HSP70 mRNA expression was increased by a 60 s, but not by a 15 or 30 s exposure at $+22.5G_z$. The expression of HSP70 followed a longer time course. These data further suggest that both *c-fos* mRNA and HSP70 mRNA may be suitable biomarkers for future investigations on post-G_z exposure effects on the rat brain.

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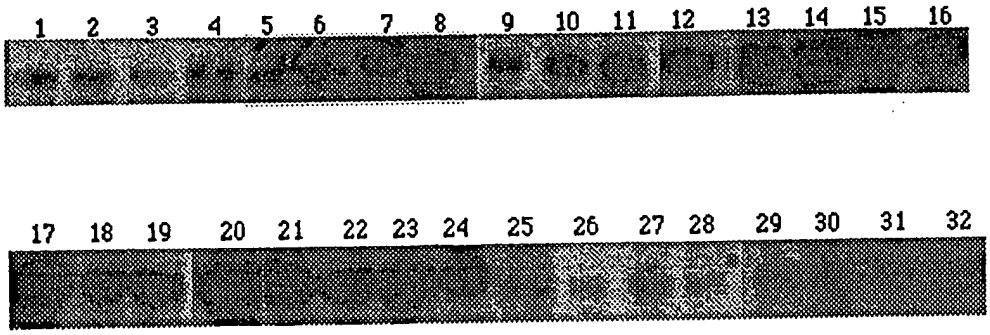
Figure 1a & 1b Typical Northern blots of c-fos and B actin mRNA



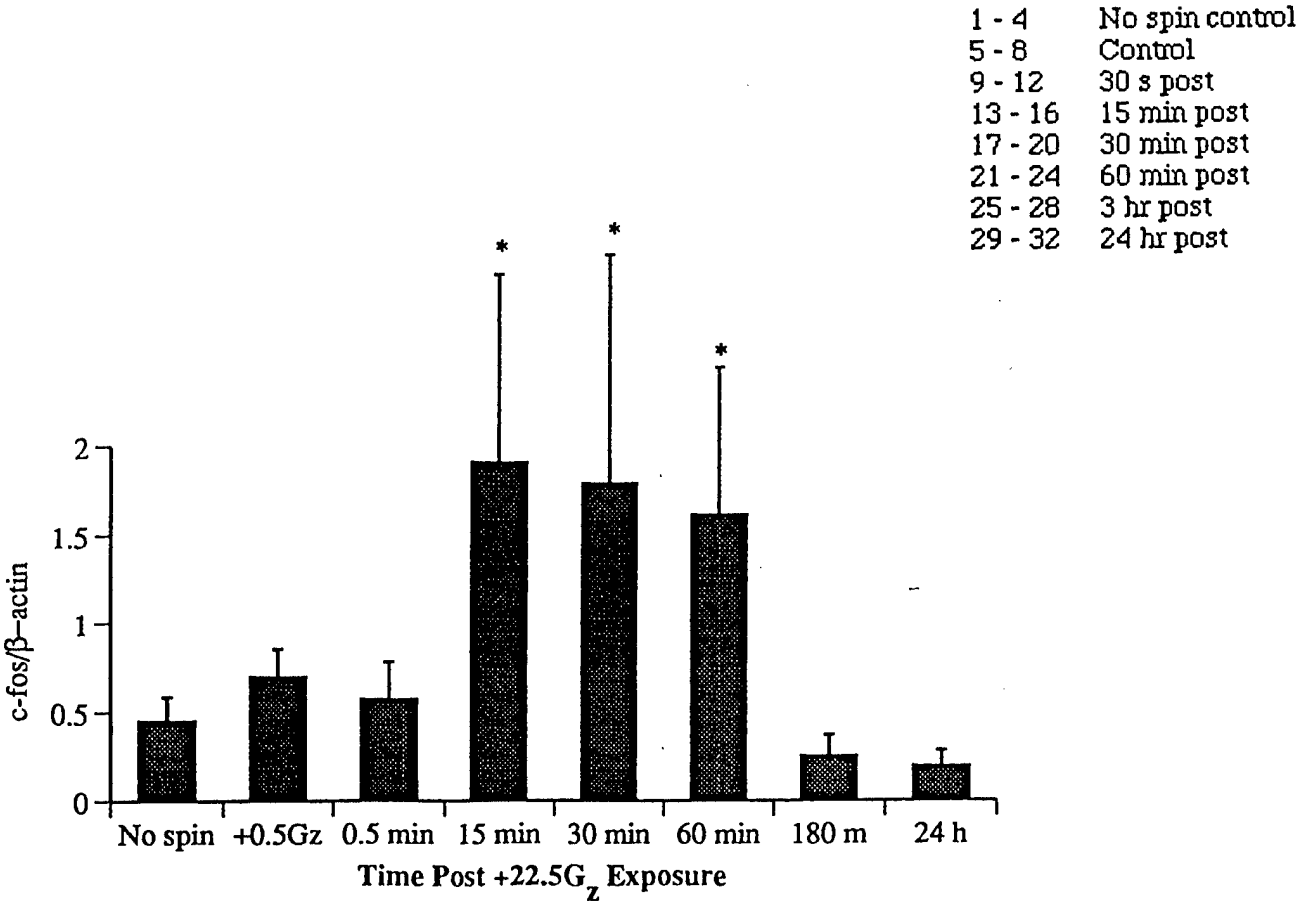
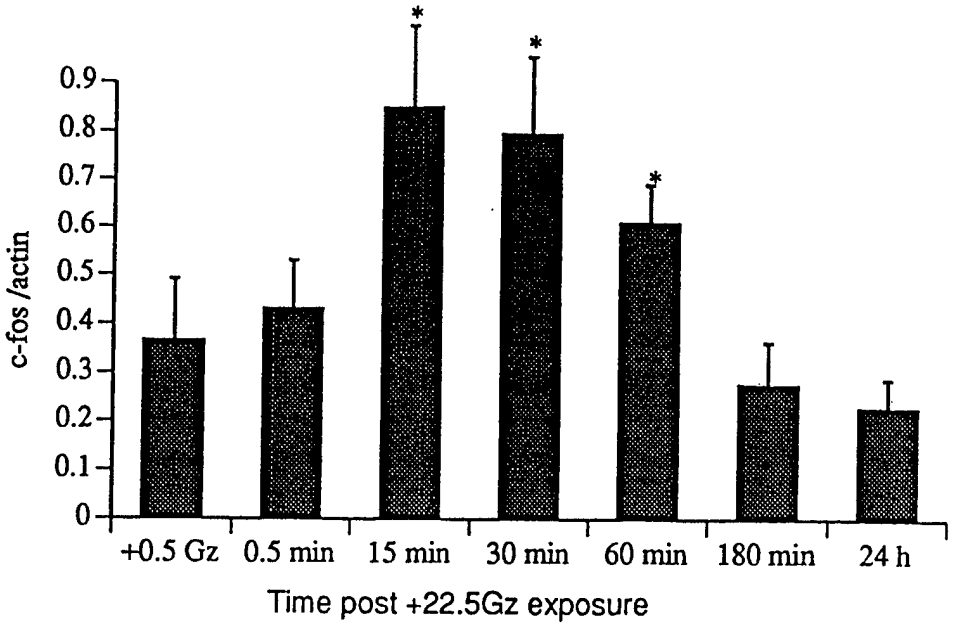


Figure 1c *c-fos* production following single +22.5Gz exposure



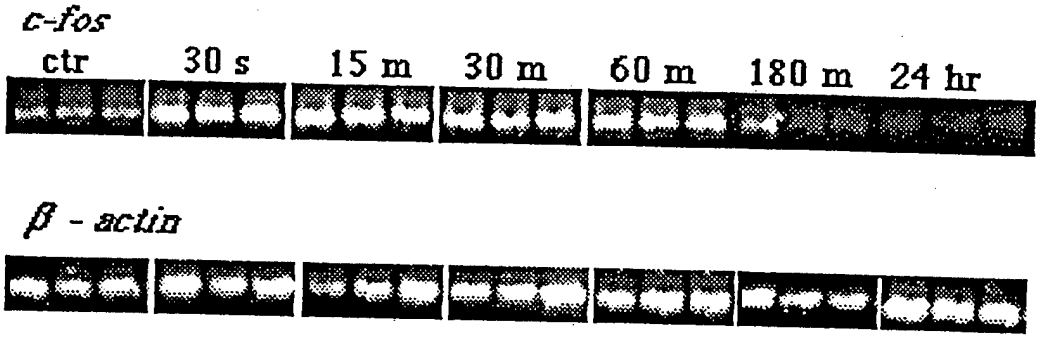


Figure 2 *c-fos* production following single +22.5Gz exposure measured by RT-PCR

Figure 3a

Northern Blot Analysis of HSP70 mRNA in Rat Brain
Following a 60 s Exposure at +22.5Gz

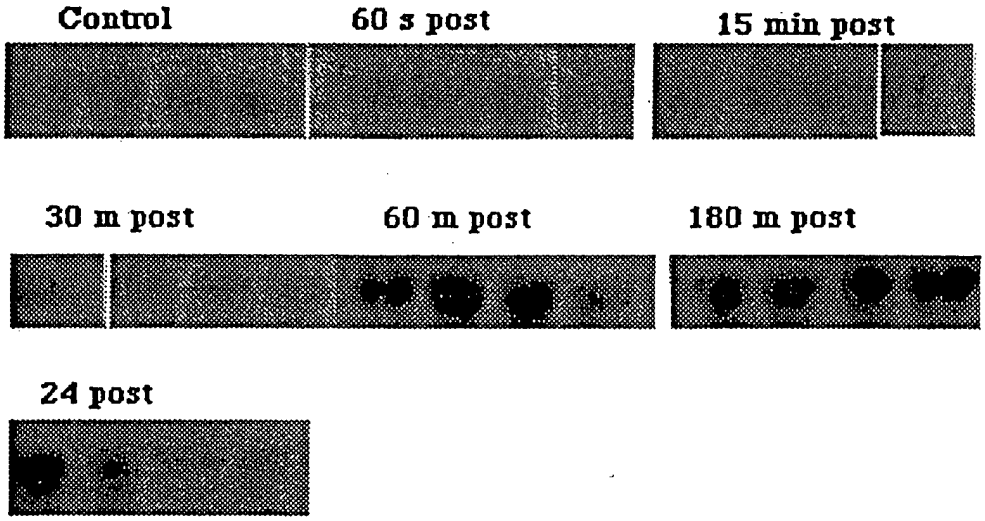
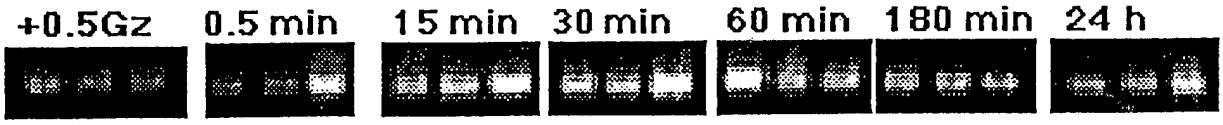


Figure 3b Expression of HSP 70 mRNA measured by RT-PCR



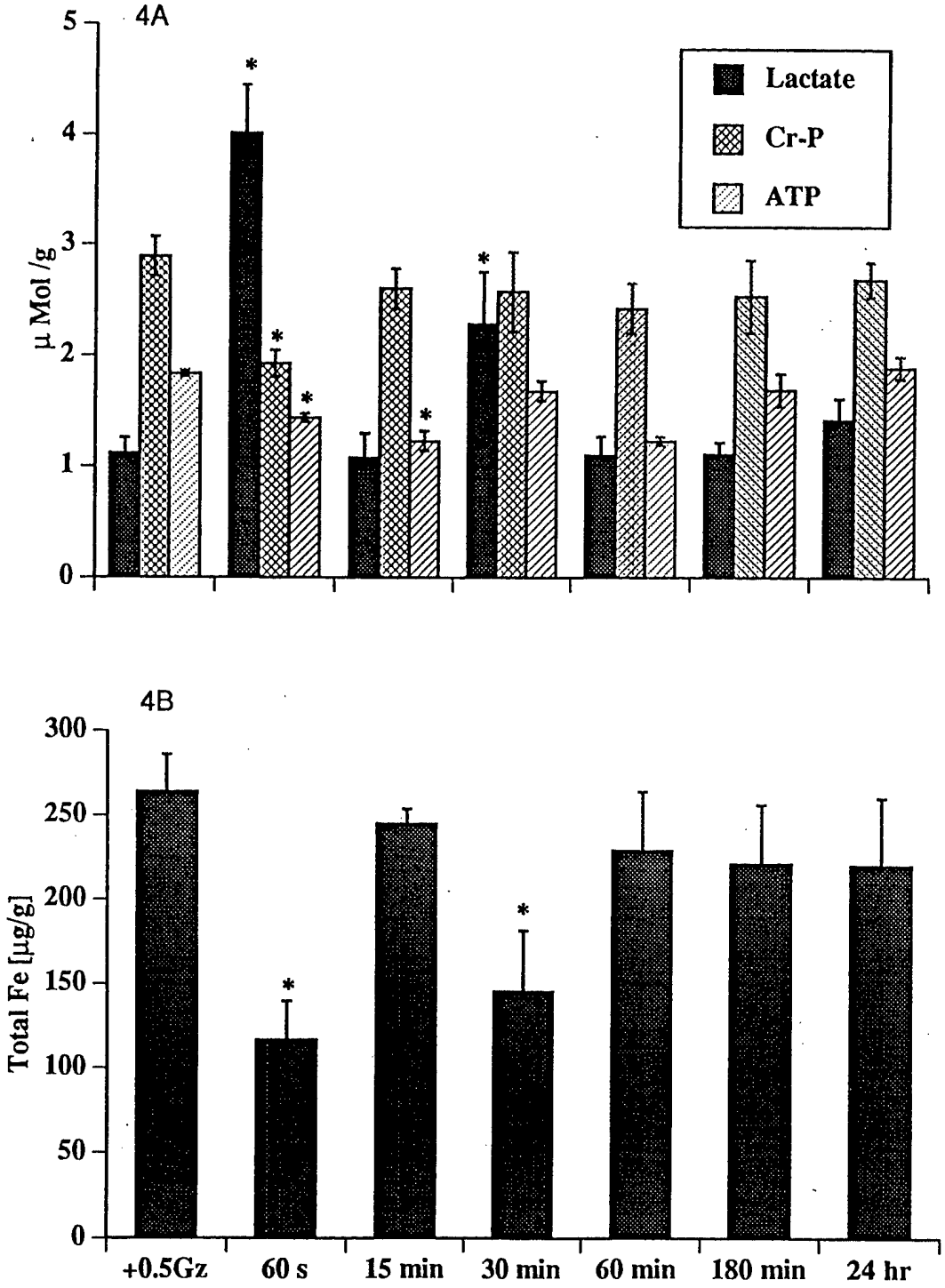


Figure 4a & 4b Effect of +22.5Gz exposure on rat brain metabolites